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Description

The invention relates to a process for protecting plant cells and plants against the action of glutamine synthetase inhibitors.

It also relates to applications of such process, particularly to the development of herbicide resistance into determined plants.

It relates further to non-biologically transformed plant cells and plants displaying resistance to glutamine synthetase inhibitors as well as to suitable DNA fragments and recombinants containing nucleotide sequences encoding resistance to glutamine synthetase inhibitors.

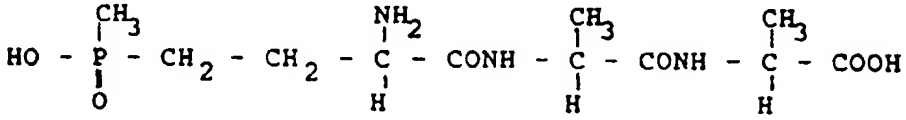
Glutamine synthetase (hereafter simply designated by GS) constitutes in most plants one of the essential enzymes for the development and life of plant cells. It is known that GS converts glutamate into glutamine. GS is involved in an efficient pathway (the only one known nowadays) in most plants for the detoxification of ammonia released by nitrate reduction, aminoacid degradation or photorespiration. Therefore potent inhibitors of GS are very toxic to plant cells. A particular class of herbicides has been developed, based on the toxic effect due to inhibition of GS in plants.

These herbicides comprise as active ingredient a GS inhibitor.

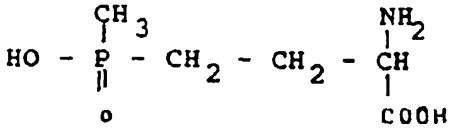
There are at least two possible ways which might lead to plants resistant to the inhibitors of the action of glutamine synthetase ; (1) by changing the target. It can be envisaged that mutations in the GS enzyme can lead to insensitivity towards the herbicide ; (2) by inactivation of the herbicide. Breakdown or modification of the herbicide inside the plant could lead to resistance.

Bialaphos and phosphinothricin (hereafter simply designated by PPT) are two such inhibitors of the action of GS, (ref. 16, 17) and have been shown to possess excellent herbicidal properties (see more particularly ref. 2 as concerns Bialaphos).

Bialaphos has the following formula (I) :



PPT has the following formula (II) :



Thus the structural difference between PPT and Bialaphos resides in the absence of two alanine aminoacids in the case of PPT.

These two herbicides are non selective. They inhibit growth of all the different species of plants present on the soil, accordingly cause their total destruction.

Bialaphos was first disclosed as having antibiotic properties, which enabled it to be used as a pesticide or a fungicide. Bialaphos can be produced according to the process disclosed in united-states patent n° 3 832 394, assigned to MEIJI SEIKA KAISHA LTD. It comprises cultivating Streptomyces hygroscopicus, such as the strain available at the American Type Culture Collection, under the ATCC number 21,705, and recovering Bialaphos from its culture medium. However, other strains, such as Streptomyces viridochromogenes, also produce this compound (ref. 1).

Other tripeptide antibiotics which contain a PPT moiety are or might be discovered in nature as well, e.g. phosalacin (ref. 15).

PPT is also obtained by chemical synthesis and is commercially distributed by the industrial Company HOECHST.

A number of Streptomyces species have been disclosed which produce highly active antibiotics which are known to incapacitate procaryotic cell functions or enzymes. The Streptomyces species which produce these antibiotics would themselves be destroyed if they had not a self defence mechanism against these antibiotics. This self defence mechanism has been found in several instances to comprise an enzyme capable of inhibiting the antibiotic effect, thus of avoiding autotoxicity for the Streptomyces species

concerned. This modification is generally reversed when the molecule is exported from the cell.

The existence of a gene which encodes an enzyme able to modify the antibiotic so as to inhibit the antibiotic effect against the host has been demonstrated in several Streptomyces producing antibiotics, for example, in S. fradiae, S. azureus, S. vinaceus, S. erythreus, producing neomycin, thiostrepton, viomycin, and MLS (Macrolide Lincosamide Streptogramin) antibiotics respectively (ref. 4), (ref. 5), (ref. 6),(ref.14 by CHATER et al., 1982 describes standard techniques which can be used for bringing these effects to light).

An object of the invention is to provide a new process for controlling the action in plant cells and plants of GS inhibitors.

Another object of the invention is to provide DNA fragments and DNA recombinants, particularly modified vectors containing said DNA fragments, which DNA fragments contain nucleotide sequences able, when incorporated in plant cells and plants, to protect them against the action of GS inhibitors.

A further object of the invention is to provide non-biologically transformed plant cells and plants capable of neutralizing or inactivating GS inhibitors.

A further object of the invention is to provide a process for selectively protecting plant species against herbicides of a GS inhibitor type.

More specifically an object of the invention is to provide a DNA fragment transferable to plant cells- and to whole plants- capable of protecting them against the herbicidal effects of Bialaphos and of structurally analogous herbicides.

The DNA fragment according to the invention, for the subsequent transformation of plant cells, consists of a nucleotide sequence coding for at least part of a polypeptide having the following sequence :

V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N F

R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

H L L K S M E A Q G F K S V V A V I

G L P N D P S V R L H E A L G Y T A

R G T L R A A G Y K H G G W H D V G

F W Q R D F E L P A P P R P V R P V

T Q I *

which part of said polypeptide is of sufficient length to confer protection against Bialaphos-"plant-protecting-capability"-, to plant cells, when incorporated genetically and expressed therein. Reference will also be made hereafter to the "plant-protecting-capability" against Bialaphos of the abovesaid nucleotide sequence.

Meaning of the designation of amino acids by a single letter is given therafter.

Alanine	A	Leucine	L
Arginine	R	Lysine	K
Asparagine	N	Methionine	M
Aspartic Acid	D	Phenylalanine	F
Cysteine	C	Proline	P
Cystine	C	Serine	S
Glycine	G	Threonine	T
Glutamic Acid	E	Tryptophan	W
Glutamine	Q	Tyrosine	Y
Histidine	H	Valine	V
Isoleucine	I		

A preferred DNA fragment consists of the following nucleotide sequence :

TAAAGAGGTGCCCGCCACCCGCTTTCGCAGAACACCGAAGGAGACCACAC

GTGAGCCCAGAACGACGCCCGGTCGAGATCCGTCCCGCCACCGCCGCCGA

CATGGCGGCGGTCTGCGACATCGTCAATCACTACATCGAGACGAGCACGG

TCAACTTCCGTACGGAGCCGCAGACTCCGCAGGAGTGGATCGACGACCTG

GAGCGCCTCCAGGACCGCTACCCCTGGCTCGTCGCCGAGGTGGAGGGCGT

CGTCGCCGGCATCGCCTACGCCGGCCCCTGGAAGGCCCGCAACGCCTACG

ACTGGACCGTCGAGTCGACGGTGTACGTCTCCCACCGGCACCAGCGGCTC

GGACTGGGCTCCACCCTCTACACCCACCTGCTGAAGTCCATGGAGGCCCA

GGGCTTCAAGAGCGTGGTCGCCGTCATCGGACTGCCCAACGACCCGAGCG

TGCGCCTGCACGAGGCGCTCGGATACACCGCGCGCGGGACGCTGCGGGCA

GCCGGCTACAAGCACGGGGGCTGGCACGACGTGGGGTTCTGGCAGCGCGA

CTTCGAGCTGCCGGCCCCGCCCCGCCCCGTCCGGCCCGTCACACAGATCT

GAGCGGAGAGCGCATGGC

or of a part thereof expressing a polypeptide having plant protecting capability against Bialaphos.

A further object of the invention is to provide plant cells resistant to the products of the class examplified by Bialaphos, which products possess the PPT unit in their structure.

The process according to the invention for controlling the action in plant cells and plants of a GS

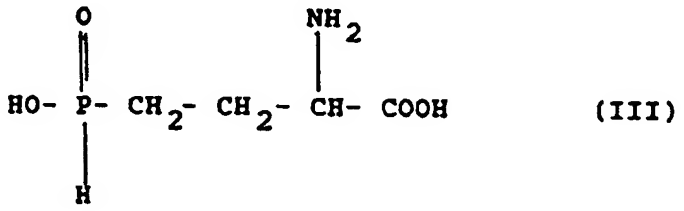
inhibitor when contacted therewith, comprises providing said plants, with the abovesaid DNA fragment which is heterologous to the plant cell genome, capable of being expressed in the form of a protein in said plant cells and plants, under condition such as to cause said heterologus DNA fragment to be integrated stably through generations in the cells of said plants, and wherein said protein has an enzymatic activity capable of inactivating or neutralization of said glutamine synthetase inhibitor.

It will be understood that the man skilled in the art should be capable of readily assessing those parts of the nucleotide sequences that could be removed from either side of any of the DNA fragments according to the invention, for instance by removing terminal parts on either side of said DNA fragment, such as by an exonucleolytic enzyme, for instance Bal31, by recloning the remaining fragment in a suitable plasmid and by assaying the capacity of the modified plasmid to transform appropriate cells and to protect it against the Bialaphos antibiotic or herbicide as disclosed later, whichever assay is appropriate.

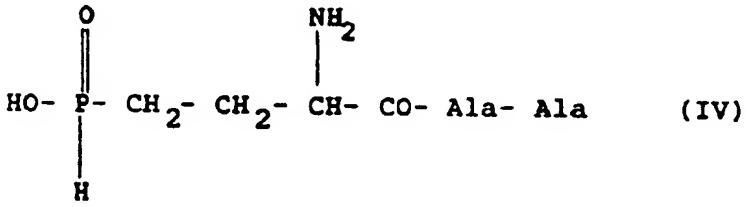
For the easiness of language, these DNA fragments will be termed hereafter as "Bialaphos-resistance DNA". In a similar manner, the corresponding polypeptide will be termed as "Bialaphos-resistance enzyme".

While in the preceding discussion particular emphasis has been put on DNA fragments capable, when introduced into plant cells and plants, to confer on them protection against Bialaphos or PPT, it should be understood that the invention should in no way be deemed as limited thereto.

In a same manner, the invention pertains to DNA fragments which, when introduced into such plant cells, would also confer on them a protection against other GS inhibitors, for instance of intermediate products involved in the natural biosynthesis of phosphinotricin, such as the compounds designated by the abbreviations MP101 (III), MP102 (IV), the formula of which are indicated hereafter :



(III)



(IV)

More generally, the invention has opened the route to the production of DNA fragments which, upon proper incorporation into plant cells and plants, can protect them against GS inhibitors when contacted therewith, as this will be shown in a detailed manner in relation to Bialaphos and PPT in the examples which will follow.

Therefore, it should be understood that the language "Bialaphos-resistance DNA" or "Bialaphos-resistance enzyme" used thereafter as a matter of convenience is intended to relate not only to the DNAs and enzymes specifically concerned with resistance to PPT or most directly related derivatives, but more generally with other DNAs and enzymes which would be capable, under the same circumstances, of inactivating the action in plants of GS inhibitors.

The invention also relates to DNA recombinants containing the above defined Bialaphos-resistance DNA fragments recombined with heterologous DNA, said heterologous DNA containing regulation elements and said Bialaphos-resistance DNA being under the control of said regulation elements in such manner as to be expressible in a foreign cellular environment compatible with said regulation elements. Particularly the abovesaid Bialaphos-resistance-DNA fragments contained in said DNA recombinants are devoid of any DNA region involved in the biosynthesis of Bialaphos, when said Bialaphos-resistance-DNA fragment originate themselves from Bialaphos-producing strains.

By "heterologous DNA" is meant a DNA of an other origin than that from which said Bialaphos-

resistance-DNA originated, e.g. is different from that of a Streptomyces hygroscopicus or Streptomyces viridochromogenes or even more preferably a DNA foreign to Streptomyces DNA. Particularly said regulation elements are those which are capable of controlling the transcription and translation of DNA sequences normally associated with them in said foreign environment. "Cellular" refers both to micro-organisms and to cell cultures.

This heterologous DNA may be a bacterial DNA, particularly when it is desired to produce a large amount of the recombinant DNA, such as for amplification purposes. In that respect a preferred heterologous DNA consists of DNA of E. coli or of DNA compatible with E. coli. It may be DNA of the same origin as that of the cells concerned or other DNA, for instance viral or plasmidic DNA known as capable of replicating in the cells concerned.

Preferred recombinant DNA contains heterologous DNA compatible with plant cells, particularly Ti-plasmid DNA.

Particularly preferred recombinants are those which contain the abovesaid GS inhibitor inactivating DNA fragment under the control of a promoter recognized by plant cells, particularly those plant cells on which inactivation of GS inhibitors is to be conferred.

Preferred recombinants according to the invention further relate to modified vectors, particularly plasmids, containing said GS-inhibitor-inactivating DNA fragment so positioned with respect to regulation elements, including particularly promoter elements, that they enable said GS inhibitor-inactivating DNA fragment to be transcribed and translated in the cellular environment which is compatible with said heterologous DNA. Advantageous vectors are those so engineered as to cause stable incorporation of said GS inhibitor inactivating DNA fragment in foreign cells, particularly in their genomic DNA. Preferred modified vectors are those which enable the stable transformation of plant cells and which confer to the corresponding cells, the capability of inactivating GS inhibitors.

It seems that, as described later, the initiation codon of the Bialaphos-resistance-gene of the Streptomyces viridochromogenes strain used herein is a GTG codon. But in preferred recombinant DNAs or vectors, the Bialaphos-resistance-gene is modified by substitution of an ATG initiation codon for the initiation codon GTG, which ATG enables translation initiation in plant cells.

In the example which follows, the plant promoter sequence which has been used was constituted by a promoter of the 35 S cauliflower mosaic virus. Needless to say that the man skilled in the art will be capable of selecting other plant promoters, when more appropriate in relation to the plant species concerned.

According to another preferred embodiment of the invention, particularly when it is desired to achieve transport of the enzyme encoded by the Bialaphos-resistance-DNA into the chloroplasts, the heterologous DNA fragment is fused to a gene or DNA fragment encoding a transit peptide, said last mentioned fragment being then intercalated between the GS inhibitor inactivating gene and the plant promoter selected.

As concerns means capable of achieving such constructions, reference can be made to the following British applications 84 32757 filed on December 28, 1984 and 85 00336 filed on January 7, 1985 and to the related applications filed in the United-States of America (n° 755,173, filed on July 15, 1985), in the European Patent Office (n° 85 402596.2 (EP-A-189 707), filed on December 20,1985), in Japan (n° 299 730, filed on December 27, 1985), in Israel (n° 77 466 filed on December 27, 1985) and in Australia (n° 5 165 485, filed on December 24, 1985).

Reference can also be made to the scientific literature, particularly to the following articles :

- VAN DEN BROECK et al., 1985, Nature, 313, 358-363 ;
- SCHREIER and al., Embo. J., vol. 4, n° 1, 25-32.

For the sake of the record, be it recalled here that under the expression "transit peptide", one refers to a polypeptide fragment which is normally associated with a chloroplast protein or a chloroplast protein subunit in a precursor protein encoded by plant cell nuclear DNA. The transit peptide then separates from the chloroplast protein or is proteolytically removed, during the translocation process of the latter protein into the chloroplasts. Examples of suitable transit peptides are those associated with the small subunit of ribulose-1,5 biphosphate (RuBP) carboxylase or that associated with the chlorophyl a/b binding proteins.

There is thus provided DNA fragments and DNA recombinants which are suitable for use in the process defined hereafter.

More particularly the invention also relates to a process, which can be generally defined as a process for producing plants and reproduction material of said plants including a heterologous genetic material stably integrated therein and capable of being expressed in said plants or reproduction material in the form of a protein capable of inactivating or neutralizing the activity of a glutamine synthetase-inhibitor, comprising the non biological steps of producing plant cells or plant tissue including said heterologous genetic material from starting plant cells or plant tissue not able to express that inhibiting or neutralizing activity,

regenerating plants or reproduction material of said plants or both from said plant cells or plant tissue including said genetic material and, optionally, biologically replicating said last mentioned plants or reproduction material or both, wherein said non-biological steps of producing said plant cells or plant tissue including said heterologous genetic material, comprises transforming said starting plant cells or plant tissue with a DNA-recombinant containing a nucleotide sequence encoding said protein and corresponding to that of the DNA fragment of the invention, as well as the regulatory elements selected among those which are capable of enabling the expression of said nucleotide sequence in said plant cells or plant tissue, and to cause the stable integration of said nucleotide sequence in said plant cells and tissue, as well as in the plant and reproduction material processed therefrom throughout generations.

The invention also relates to the cell cultures containing said Bialaphos-resistance-DNA fragment, which cell cultures have the property of being resistant to a composition containing a GS inhibitor, when cultured in a medium containing a such composition at dosages which would be destructive for non transformed cells.

The invention concerns more particularly those plant cells or cell cultures in which the Bialaphos-resistance DNA fragment of this invention is stably integrated and which remains present over successive generations of said plant cells. Thus the resistance to a GS inhibitor, more particularly Bialaphos or PPT, can also be considered as a way of characterizing the plant cells of this invention.

Optionally one may also resort to hybridization experiments between the genomic DNA obtained from said plant cells with a probe containing a GS inhibitor inactivating DNA sequence.

More generally the invention relates to plant cells, reproduction material, particularly seeds, as well as plants containing said foreign or heterologous DNA fragment stably integrated in their respective genomic DNAs, said fragments being transferred throughout generations of such plant cells, reproduction material, seeds and plants, wherein said DNA fragment encodes a protein inducing a non-variety-specific enzymatic activity capable of inactivating or neutralizing GS inhibitors, particularly Bialaphos and PPT, more particularly to confer on said plant cells, reproduction material, seeds and plants a corresponding non-variety-specific phenotype of resistance to GS inhibitors.

"Non-variety-specific" enzymatic activity or phenotype aims at referring to the fact that they are not characteristic of specific plant genes or species as this will be illustrated in a non-limitative way by the examples which will follow. They are induced in said plant materials by essentially non-biological processes applicable to plants belonging to species normally unrelated with one another and comprising the incorporation into said plant material of heterologous DNA, e.g. bacterial DNA or chemically synthesized DNA, which does not normally occur in said plant material or which normally cannot be incorporated therein by natural breeding processes, and which yet confers a common phenotype (e.g. herbicide resistance) to them.

The invention is of particular advantageous use in processes for protecting field-cultivated plant species against weeds, which processes comprise the step of treating the field with an herbicide, e.g. Bialaphos or PPT in a dosage effective to kill said weeds, wherein the cultivated plant species then contains in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing or inactivating said GS inhibitor.

By way of illustration only, effective doses for use in the abovesaid process range from about 0.4 to about 1.6 kg/Hectare of Bialaphos or PPT.

There follows now a disclosure of how the preferred DNA fragment, the "sfrsv" gene described hereabove, was isolated starting from the Streptomyces viridochromogenes strain, the construction of expression vectors which contain the resistance gene and which allow the subsequent transformation of plant cells, in order to render them resistant to GS inhibitors.

The disclosure will then be completed with the description of the insertion of a preferred DNA fragment conferring to the transformed cells the capability of inactivating Bialaphos and PPT. Thus the Bialaphos-inactivating-DNA fragment designated thereafter by Bialaphos-resistance gene or "sfrsv" gene, isolated by the above described technique into plasmids which can be used for transforming plant cells and conferring to them a resistance against Bialaphos, also merely by way of example for non-limitative illustration purposes.

The following disclosure is made with reference to the description of the earlier EP-A-242236 as applied to another gene, the "sfr" gene, obtained from a Streptomyces hygroscopicus strain available at the American Type Culture Collection under deposition number ATCC 21 705, since procedures have much in common. Particularly as shown from the description which follows, much of the materials used in construction using the "sfr" gene happened to be immediately transposable to the "sfrsv" gene. Since the description of the "sfrsv"-based constructions often refer to the earlier description of the "sfr"-based constructions, it is proposed for the sake of clarity and of full availability to the public, to retain all examples

disclosed in European Patents in respect of the "sfr" gene as a supplementation of the description of all possible uses of the "sfrsv" gene, which is more particularly the subject of the present application. Reference will also be made to the drawings in which:

- fig. 1 is a restriction map of a plasmid containing a Streptomyces hygroscopicus DNA fragment encoding Bialaphos-resistance, which plasmid, designated hereafter as pBG1 has been constructed according to the disclosure which follows ;
- fig. 2 shows the nucleotide sequence of a smaller fragment obtained from pBG1, subcloned into another plasmid (pBG39) and containing the resistance gene ;
- fig. 3 shows the construction of a series of plasmids given by way of example, which plasmids aim at providing suitable adaptation means for the insertion therein of the Bialaphos-resistance gene or "sfr" gene ;
- fig. 4A and 4B show the construction of a series of plasmids given by way of example, which plasmids contain suitable plant cell promoter sequences able to initiate transcription and expression of the foreign gene inserted under their control into said plasmids ;
- fig. 5A shows a determined fragment of the nucleotide sequence of the plasmid obtained in figure 3 ;
- fig. 5B shows the reconstruction of the first codons of a Bialaphos-resistance gene, from a FokI/BglII fragment obtained from pBG39 and the substitution of an ATG initiation codon for the GTG initiation codon of the natural "sfr" gene ;
- fig. 5C shows the reconstruction of the entire "sfr" gene, namely the last codons thereof, and its insertion into a plasmid obtained in figures 4A and 4B ;
- fig. 6A shows an expression vector containing the "sfr" gene placed under the control of a plant cell promoter ;
- fig. 6B shows another expression vector derived from the one shown in fig. 6A, by the substitution of some nucleotides.
- fig. 7 shows the construction of a series of plasmids given by way of examples, to ultimately produce plasmids containing the promoter region and the transit peptide sequence of a determined plant cell gene, for the insertion of the "sfr" gene under the control of said promoter region and downstream of said transit peptide sequence.
- fig. 8 shows the restriction map of a plasmid pJS1 containing another Bialaphos-resistance-gene;
- fig. 9 shows the nucleotide sequence of the "sfrsv" gene containing the resistance gene;
- fig. 10 shows the amino acid homology of "sfrsv" gene and "sfr" gene,
- fig. 11 shows the construction of plasmid, given by way of example; which contains the "sfrsv" gene and suitable for the transformation of plant cells.

The Bialaphos-resistance-gene isolated from Streptomyces viridochromogenes, is thereafter designated by "sfrsv" gene.

The following experiment was set up to isolate a Bialaphos-resistance-gene from S. hygroscopicus, according to standard techniques for cloning into Streptomyces.

2.5 µg of S. hygroscopicus (EP-A-242 236) genomic DNA and 0.5 µg of Streptomyces vector pIJ61 were cleaved with PstI according to the method described in ref. 6. The vector fragments and genomic fragments were mixed and ligated (4 hours at 10°C followed by 72 hours at 4°C in ligation salts which contain 66 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 0.1 mM ATP) at a total DNA concentration of 40 µg ml^{-1} with T4 DNA ligase. Ligation products were introduced into 3 x 10^9 S. lividans strain 66 protoplasts by a transformation procedure mediated by polyethylene-glycol (PEG) as described hereafter. These protoplasts gave rise to 5 x 10^7 colonies and 4 x 10^4 pocks after regeneration on 20 plates of R2 agar containing 0.5 % of Difco yeast extract (R2 YE). Preparation and composition of the different mediums and buffers used in the disclosed experiments are described hereinafter. When these lawns were replica-plated on minimal medium plates containing 50 µg ml^{-1} Bialaphos, drug resistant colonies appeared at a frequency of 1 per 10^4 transformants. After purification of the drug resistant colonies, there plasmid DNA was isolated and used to retransform S. lividans protoplasts. Non selective regeneration followed by replication to Bialaphos-containing-medium demonstrated a 100 % correlation between pocks and Bialaphos resistant growth. The recombinant plasmids of several resistant clones all contained a 1.7 Kb PstI insert (see fig. 1).

Subcloning of the herbicide resistance gene

The 1.7 Kb PstI insert was then subcloned into the high copy number streptomycete vector pIJ385 to generate plasmid pBG20. S. lividans strains which contained pBG20 were more than 500 times more resistant to Bialaphos. S. lividans growth is normally inhibited in minimal medium containing 1 µg/ml

Bialaphos ; growth of transformants containing pBG20 was not noticeably inhibited in a medium containing 500 µg/ml Bialaphos. The PstI fragment was also subcloned in either orientation into the PstI site of the plasmid pBR322, to produce plasmids pBG1 and pBG2, according to their orientation. A test on minimal M9 medium demonstrated that E. coli E8767 containing pBG1 or pBG2 was resistant to Bialaphos.

A ± 1.65 Kb PstI - BamHI fragment was subcloned from pBG1 into the plasmid pUC19 to produce the plasmid pBG39, and conferred Bialaphos resistance to E. coli, W3110, C600 and JM83.

Using an in vitro coupled transcription-translation system (ref. 5) from S. lividans extracts, the 1,65 Kb PstI - BamHI fragment in pBG39 was shown to direct the synthesis of a 22 Kd protein. In the following, this 1,65 Kb insert includes a fragment coding for a 22 Kd protein and will be called "sfr" gene.

Fine mapping and sequencing of the gene

A 625 bp Sau3A fragment was subcloned from pBG39 into pUC19 and still conferred Bialaphos resistance to a E. coli W3110 host. The resulting clones were pBG93 and pBG94, according to the orientation.

The orientation of the gene in the Sau3A fragment was indicated by experiments which have shown that Bialaphos resistance could be induced with IPTG from the pUC19 lac promoter in pBG93. In the presence of IPTG (0.5 mM) the resistance of pBG93/W3110 increased from 5 to 50 µg/ml on a M9 medium containing Bialaphos. The W3110 host devoid of pBG93, did not grow on M9 medium containing 5 µg/ml Bialaphos. These experiments demonstrated that the Sau3A fragment could be subcloned without loss of activity. They also provided for the proper orientation as shown in the fig. 2, enclosed thereafter. The protein encoded by these clones was detected by using coupled transcription-translation systems derived from extracts of S. lividans (ref. 7). Depending on the orientation of the Sau3A fragment, translation products of different sizes were observed ; 22 Kd for pBG94 and ± 28 Kd for pBG93. This indicated that the Sau3A fragment did not contain the entire resistance gene and that a fusion protein was formed which included a polypeptide sequence resulting from the translation of a pUC19 sequence.

In order to obtain large amounts of the protein, a 1.7 Kb PstI fragment from pBG1 was cloned into the high copy number Streptomycete replicon pIJ385. The obtained plasmid, pBG20, was used to transform S. hygroscopicus. Transformants which contained this plasmid had more than 5 times as much PPT acetylating activity and also had increased amounts of a 22 kd protein on sodium dodecylsulfate gels (SDS gels). Furthermore, both the acetyl transferase and the 22 kd protein appeared when the production of Bialaphos begun. The correlation of the in vitro data, kinetics of synthesis, and amplified expression associated with pBG20 transformants strongly implied that this 22 Kd band was the gene product.

The complete nucleotide sequence of the 625 bp Sau3A fragment was determined as well as of flanking sequences. Computer analysis revealed the presence of an open reading frame over the entire length of the Sau3A fragment.

Characterization of the sfr gene product

A series of experiments were performed to determine that the open reading frame of the "sfr" gene indeed encoded the Bialaphos resistance enzyme. To determine the 5' end of the resistance gene, the NH_2-terminal sequence of the enzyme was determined. As concerns more particularly the technique used to determine the said sequence, reference is made to the technique developed by J. VANDEKERCKHOVE, Eur. J. Bioc. 152, p. 9-19, 1985, and to French patent applications n° 85 14579 filed on October 1st, 1985 and n° 85 13046 filed on September 2nd, 1985, all of which are incorporated herein by reference.

This technique allows the immobilization on glass fibre sheets coated with the polyquaternary amine commercially available under the registered trademark POLYBRENE of proteins and of nucleic acids previously separated on a sodium dodecylsulfate containing polyacrylamide gel. The transfer is carried out essentially as for the protein blotting on nitrocellulose membranes (ref. 8). This allows the determination of amino-acid composition and partial sequence of the immobilized proteins. The portion of the sheet carrying the immobilized 22 kd protein produced by S. hygroscopicus pBG20 was cut out and the disc was mounted in the reaction chambre of a gas-phase sequenator to subject the glass-fibre bound 22 Kd protein to the Edman degradation procedure. The following amino-acid sequence was obtained :

Pro-Glu-Arg-Arg-Pro-Ala-Asp-Ile-Arg-Arg

This sequence matched an amino-acid sequence which was deduced from the open reading frame of the 625 bp Sau3A fragment. It corresponded to the stretch from codon 3 to codon 12.

Thus, the NH_2-terminus of the 22 Kd protein was upstream of this sequence. It was determined that translation of the actual protein was likely to be initiated 2 amino-acids earlier at a GTG initiation codon.

GTG is often used as initiator codon in Streptomyces and translated as methionine. The protein translated from the GTG initiation codon would be 183 amino-acids long and would have a molecular weight of 20 550. This was in good agreement with the observed approximate molecular weight of 22 000.

Furthermore, the termination codon, TGA, was located just downstream of the Sau3A site. Cloning of the 625 bp Sau3A fragment in a BamHI site digested pUC19 did not result in the reconstruction of the termination codon. This explained the fusion proteins which were observed in the in vitro transcription-translation analysis.

Mechanism of PPT-resistance

Having defined a first phenotype and some of the physical characteristics of the resistance gene and its gene product, a series of experiments was then carried out to understand the mechanism by which it confers resistance. As described hereabove, PPT is the portion of Bialaphos which inhibits glutamine synthetase (GS) and that N-acetyl PPT is not an inhibitor. Using a standard assay (ref. 9), S. hygroscopicus ATCC 21 705 derivates were shown to contain a PPT acetyl transferase which was not found in S. lividans. The activity does not acetylate the Bialaphos tripeptide. S. lividans carrying the resistance gene cloned in pBG20 or pBG16 (a plasmid containing the 625 bp Sau3A fragment cloned into another streptomycete vector, pIJ680) also contained the activity which could acetylate PPT but not Bialaphos. The PPT derived reaction product produced by extracts of pBG20/S. lividans was isolated in order to confirm that it was indeed acetyl-PPT. Analysis by mass spectroscopy showed that the molecular weight had increased relative to PPT by the equivalent of one acetyl group. It was thus concluded that the 625 bp Sau3A fragment contained sequences which code for PPT acetyl transferase.

The experimental conditions and reagents used in the techniques disclosed hereabove were as follows :

Preparation and composition of the mediums and buffers above used

1° P medium : 10.3 g of sucrose, 0.025 g of K_2SO_4,0.203 g of $MgCl_2.6H_2O$ and 0.2 ml of a trace element solution are dissolved in 80 ml of distilled water and autoclaved. Then in order, 1 ml of KH_2PO_4 (0.5%), 10 ml of $CaCl_2$, $2H_2O$ (3.68 %), and 10 ml of TES buffer (0.25 M), pH : 7.2) are added. Trace element solution (per litre) : $ZnCl_2$, 40 mg ; $FeCl_3.6H_2O$, 200 mg ; $CuCl_2.2H_2O$, 10 mg ; $MnCl_2.4H_2O$, 10 mg ; $Na_2B_4O_7.10H_2O$, 10 mg ; $(NH_4)_6Mo_7O_{24}.4H_2O$, 10 mg.

2° R2YE : 10.3 g of sucrose, 0.025 g of K_2SO_4, 1.012 g of $MgCl_2.6H_2O$, 1 g of glucose, 0.01 g of Difco casamino acids, and 2.2 g of Difco agar are dissolved in 80 ml distilled water and autoclaved. 0.2 ml of trace element solution, 1 ml of KH_2PO_4 (0.5%), 8.02 ml of $CaCl_2.2H_2O$ (3.68 %), 1.5 ml of L-proline (20 %), 10 ml of TES buffer (0.25 M) (pH : 7.2), 0.5 ml of (1 M) NaOH, 5 ml of yeast extract (10 %) are sequentially added.

3° TE : 10 mM TRIS HCl, 1 mM EDTA, pH 8.0.

4° YEME : Difco yeast extract (0.3 %), Difco peptone (0.5 %), oxoid malt extract (0.3 %), glucose (1 %).

Transformation of S. lividans protoplasts

1. A culture composed of 25 ml YEME, 34 % sucrose, 0.005 M $MgCl_2$, 0.5 % glycine, in a 250 ml baffled flask, is centrifuged during 30 to 36 hours.
2. The pellet is suspended in 10.3 % sucrose and centrifuged. This washing is repeated once.
3. The mycelium is suspended in 4 ml lysozyme solution (1 mg/ml in P medium with $CaCl_2$ and $MgCl_2$ concentrations reduced to 0.0025 M) and incubated at 30 ° C for 15 to 60 minutes.
4. The solution is mixed by pipetting three times in a 5 ml pipette and incubated for further 15 minutes.
5. P medium (5 ml) is added and mixed by pipetting as in step 4.
6. The solution is filtered through cotton wool and protoplasts are gently sedimented in a bench centrifuge at 800 x G during 7 minutes.
7. Protoplasts are suspended in 4 ml P medium and centrifuged again.
8. Step 7 is repeated and protoplasts are suspended in the drop of P medium left after pouring off the supernatant (for transformation).
9. DNA is added in less than 20 µl TE.
10. 0.5 ml PEG 1 000 solution (2.5 g PEG dissolved in 7.5 ml of 2.5% sucrose, 0.0014 K_2SO_4, 0.1 M $CaCl_2$, 0.05 M TRIS-maleic acid, pH 8.0, plus trace elements) is immediately added and pipetted once to mix the components.
11. After 60 seconds, 5 ml of P medium are added and the protoplasts are sedimented by gentle

centrifugation.

12. The pellet is suspended in P medium (1 ml).

13. 0.1 ml is plated out on R2YE plates (for transformation dry plates to 85% of their fresh weigh e. g. in a laminar flow cabinet).

14. Incubation at 30 ° C.

A - Construction of a "sfr" gene cassette

A "sfr" gene cassette was constructed to allow subsequent cloning in plant expression vectors.

Isolation of a FokI-BglII fragment from the plasmid pBG39 containing a "sfr" gene fragment led to the loss of the first codons, including the initiation codon, and of the last codons, including the stop codon.

This fragment of the "sfr" gene could be reconstructed in vitro with synthetic oligonucleotides which encode appropriate amino-acids.

The complementary synthetic oligonucleotides were 5'-CATGAGCCCAGAAC and 3'-TCGGGTCTTGCTGC.

By using such synthetic oligonucleotides, the 5' end of the "sfr" gene could be reformed and the GTG initiation codon substituted for a codon well translated by plant cells, particularly an ATG codon.

The DNA fragment containing the oligonucleotides linked to the "sfr" gene was then inserted into an appropriate plasmid, which contained a determined nucleotide sequences thereafter designated by an "adapter" fragment.

This adapter fragment comprised :

- a TGA termination codon which enabled the last codons of the "sfr" gene to be reformed ;
- appropriate restriction sites which enabled the insertion of the fragment of the nucleotide sequence comprising the "sfr" gene partially reformed with the synthetic oligonucleotides ; this insertion resulted in the reconstruction of an intact "sfr" gene ;
- appropriate restriction sites for the isolation of the entire "sfr" gene.

The "sfr" gene was then inserted into another plasmid, which contained a suitable plant promoter sequence. The plant promoter sequence consisted of the cauliflower mosaïc virus promoter sequence (p35S). Of course the invention is not limited to the use of this particular promoter. Other sequences could be chosen as promoters suitable in plants, for example the TR 1'-2' promoter region and the promoter fragment of a Rubisco small subunit gene from Arabidopsis thaliana hereafter described.

1° Construction of the plasmid pLK56.2 (fig. 3)

The construction of plasmid pLK56.2 aimed at obtaining a suitable adaptor including the following sequence of restriction sites : SmaI, BamHI, NcoI, KpnI, BglII, MluI, BamHI, HindIII and XbaI.

The starting plasmids used for this construction, pLK56, pJB64 and pLK33 were those disclosed by BOTTERMAN (ref. 11).

The DNA fragments hereafter described were isolated and separated from low melting point agarose (LGA).

The plasmid pLK56 was cleaved by the enzymes BamHI and NdeI. A NcoI-NdeI fragment (referred to in the drawings by arc "a" in broken line) obtained from plasmid pJB64 was substituted in pLK56 for the BamHI-NdeI fragment shown at "b". Ligation was possible after filling in the BamHI and NcoI protruding ends with the DNA polymerase I of E. coli (Klenow's fragment).

Particularly recircularization took place by means of a T4 DNA ligase. A new plasmid pLK56.3 was obtained.

This plasmid was cleaved by the enzymes XbaI and PstI.

The BamHI-PstI fragment of pLK33 (c) (on fig. 3) was substituted for the XbaI-PstI fragment (d) of pLK56.3, after repairing of their respective ends by Klenow's fragment.

After recircularization by means of the T4 DNA ligase, the obtained plasmid pLK56.2 contained a nucleotide sequence which comprised the necessary restriction sites for the subsequent insertion of the "sfr" gene.

2° Construction of the plasmid pGSH150 (fig. 4A)

Parallel with the last discussed construction, there was produced a plasmid containing a promoter sequence recognized by the polymerases of plant cells and including suitable restriction sites, downstream of said promoter sequence in the direction of transcription, which restriction sites are then intended to

enable the accomodation of the "sfr" gene then obtainable from pLK56.2, under the control of said plant promoter.

Plasmid pGV825 is described in DEBLAERE et al. (ref. 10). Plasmid pJB63 is from BOTTERMAN (ref. 11).

pGV825 was linearized with PvuII and recircularized by the T4 DNA ligase, resulting in the deletion of an internal PvuII fragment shown at (e), (plasmid pGV956).

pGV956 was then cleaved by BamHI and BglII.

The BamHI-BglII fragment (f) obtained from pJB63 was dephosphorylated with calf intestine phosphatase (CIP) and substituted for the BamHI-BglII fragment of pGV956.

Plasmid pGV1500 was obtained after recircularization by means of T4 DNA ligase.

An EcoRI-HindIII fragment obtained from plasmid pGSH50 was purified. The latter plasmid carried the dual TR 1'-2' promoter fragment described in VELTEN et al., (ref.13). This fragment was inserted in pGV1500, digested with HpaI and HindIII and yielded pGSH150.

This plasmid contains the promoter fragment in front of the 3' end of the T-DNA transcript 7 and a BamHI and ClaI sites for cloning.

3° Construction of the plasmid pGSJ260 (fig. 4B)

CP3 is a plasmid derived from pBR322 and which contains the 35S promoter region of cauliflower mosaïc virus within a BamHI fragment.

pGSH150 was cut by BamHI and BglII.

The BamHI-BglII fragment (h) of CP3, which contained the nucleotide sequence of p35S promoter, was substituted for the BamHI-BglII fragment (i) in pGSH150 to form plasmid pGSJ250. pGSJ250 was then opened at its BglII restriction site.

A BamHI fragment obtained from mGV2 (ref. 12) was inserted in pGSJ250 at the BglII site to form plasmid pGSJ260.

However prior to inserting the "sfr" gene obtainable from pLK56.2 into plasmid pGSJ260, it was still desirable to further modify the first in order to permit insertion in a more practical manner. Thus pLK56.2 was further modified as discussed below to yield pGSR1.

Starting from plasmid pGSJ260, two plasmid constructions for subsequent transformations of plant cells were made :

- a first plasmid permitting the expression of the "sfr" gene in the cytoplasm of plant cells, and
- a second plasmid so modified as to achieve transport of the Bialaphos-resistance enzymes to the chloroplasts of plant cells.

First case : plasmid enabling the expression of the "sfr" gene in the cytoplasm of plant cells

Cloning of the sfr gene cassette in a plant expression vector (pGSR2) (fig. 5)

On figure 5A, the nucleotide sequence of the adapter of pLK56.2 is shown. In particular, the locations of BamHI, NcoI, BglII restriction sites are shown.

This adapter fragment was cleaved by the enzymes NcoI and BglII.

Figure 5B shows the FokI-BglII fragment (j) obtained from pBG39. The locations of these two restriction sites are shown on figure 2.

Using synthetic oligonucleotides, the first codons of the "sfr" gene were reformed, particularly the 5' end of the gene in which a ATG initiation codon was substituted for the initial GTG codon.

This FokI-BglII fragment completed with the synthetic oligonucleotides was then substituted in pLK56.2 for the NcoI-BglII fragment of the adapter. The 3' end of the gene was thus reformed too, after recircularization with T4 DNA ligased. The plasmid obtained, pGSR1, thus contained the entire "sfr" gene inserted in its adapter.

The plasmid pGSJ260 was then opened by BamHI (fig. 5C) and the BamHI fragment obtained from pGSR1, which contained the entire "sfr" gene, was inserted into pGSJ260.

The obtained plasmid, pGSR2 (see fig. 6A) contained a pBR322 replicon, a bacterial streptomycin resistance gene (SDM-SP-AD-transferase) and an engineered T-DNA consisting of :

- the border fragments of the T-DNA ;
- a chimeric kanamycin gene which provided a dominant selectable marker in plant cells ; and
- a chimeric "sfr" gene.

The chimeric "sfr" gene consisting of :

- the promoter region of the cauliflower mosaïc virus (p35S) ;
- the "sfr" gene cassette as described in fig. 5 ;
- the 3' untranslated region, including the polyadenylation signal of T-DNA transcript 7.

pGSR2 was introduced into Agrobacterium tumefaciens recipient C58ClRif^R (pGV2260) according to the procedure described by DEBLAERE et al. (ref. 10).

This strain was used to introduce the chimeric "sfr" gene in N. tabacum SR_1 plants.

Two variant plasmids deriving from pGSR2, namely pGSFR280 and pGSFR281, have been constructed. They differ in the untranslated sequence following the transcription initiation site. In pGSR2, this fragment consists of the following sequence :

GAGGACACGCTGAAATCACCAGTCTCGGATCCATG ;

while it consists of :

GAGGACACGCTGAAATCACCAGTCTCTCTACAAATCGATCCATG

in pGSR280 and of

GAGGACACGCTGAAATCACCAGTCTCTCTACAAATCGATG

in pGSFR281, with an ATG codon being the initiation codon of the "sfr" gene. The "sfr" gene is also fused to the TR1'-2' promoter in the plasmid pGSH150 (fig. 4A) yielding pGSFR160 and pGSFR161 (fig. 6B). These plasmids contain slight differences in the pTR2 "sfr" gene configuration : the "sfr" gene is correctly fused to the endogenous gene 2' ATG in pGSFR161 (for sequences see ref. 13), whereas 4 extra base pairs (ATCC) are present just ahead of the ATG codon in pGSFR160. Otherwise, plasmids p65FR161 and p65FR160 are completely identical.

All plasmids are introduced in Agrobacterium by cointegration in the acceptor plamids pGV2260 yielding the respective plasmids pGSFR1280, pGSFR1281, pGSFR1160 and pGSFR1161.

Second case : construction of a plasmid containing the "sfr" gene downstream of a DNA sequence encoding a transit peptide and suitable for achieving subsequent translocation of the "sfr" gene expression product into plant-cell-chloroplasts

In another set of experiments, the nucleotide sequence which contained the "sfr" gene was fused to a DNA sequence encoding a transit peptide so as to enable its transport into chloroplasts.

A fragment of the "sfr" gene was isolated from the adapter fragment above described and fused to a transit peptide. With synthetic oligonucleotides, the entire "sfr" gene was reconstructed and fused to a transit peptide.

The plasmid (plasmid pATS3 mentioned below) which contained the nucleotide sequence encoding the transit peptide comprised also the promoter sequence thereof.

Construction of the plasmid pGSR4 which contains the "sfr" gene fused to a DNA sequence encoding transit peptide (fig. 7)

Plasmid pLK57 is from BOTTERMAN, (ref.11). Plasmid pATS3 is a pUC19 clone which contains a 2Kb EcoRI genomic DNA fragment from Arabidopsis thaliana comprising the promoter region and the transit peptide nucleotide sequence of the gene, the expression thereof is the small subunit of ribulose biphosphate carboxylase (ssu). The A. thaliana small subunit was isolated as a 1 500 bp EcoRI-SphI fragment. The SphI cleavage site exactly occurs at the site where the coding region of the mature ssu protein starts.

Plasmids pLK57 and pATS3 were opened with EcoRI and SphI. After recircularization by means of the T4 DNA ligase. a recombinant plasmid pLKAB1 containing the sequence encoding the transit peptide (Tp) and its promoter region (Pssu) was obtained.

In order to correctly fuse the "sfr" gene at the cleavage site of the signal peptide, the N-terminal gene sequence was first modified. Since it was observed that N-terminal gene fusions with the "sfr" gene retain their enzymatic activity, the second codon (AGC) was modified to a GAC, yielding an NcoI site overlapping with the ATG initiator site. A new plasmid, pGSSFR2 was obtained. It only differs from pGSR1 (fig. 5B), by that mutation. The NcoI-BamHI fragment obtained from pGSFR2 was fused at the SphI end of the transit peptide sequence. In parallel, the "sfr" gene fragment was fused correctly to the ATG initiator of the ssu gene (not shown in figures).

Introduction of the "sfr" gene into a different plant species

The Bialaphos-resistance induced in plants by the expression of chimeric genes, when the latter have

been transformed with appropriate vectors containing said chimeric genes, has been demonstrated as follows. The recombinant plasmids containing the "sfr" gene were introduced separately by mobilization into Agrobacterium strain C58C$_1$ Rif^R (pGV2260) according to the procedure described by DEBLAERE and al., Nucl. Acid. Res., 13, p. 1 477, 1985. Recombinant strains containing hybrid Ti plasmides were formed. These strains were used to infect and transform leaf discs of different plant species, according to a method essentially as described by HORSH and al., 1985, Science, vol. 227. Transformation procedure of these different plant species given by way of example, is described thereafter.

1. Leaf disc transformation of Nicotiana tabacum

Used Media are described thereafter :

A_1 MS salt/2	+ 1% sucrose 0.8% agar pH 5.7
A_{10} B5-medium	+ 250 mg/l NH_4NO_3 750 mg/l $CaCl_2$ $2H_2O$ 0.5 g/l 2-(N-Morpholino)ethane-sulfonic acid (MES) pH 5.7 30 g/l sucrose
A_{11} B5-medium	+ 250 mg/l NH_4NO_3 0.5 g/l MES pH 5.7 2 % glucose 0.8 % agar 40 mg/l adenine + 1 mg/l 6-Benzylaminopurine (BAP) 0.1 mg/l Indole-3-acetic acid (IAA) 500 mg/l Claforan
A_{12} B5-medium	+ 250 mg/l NH_4NO_3 0.5 g/l MES pH 5.7 2 % glucose 0.8 % agar 40 mg/l adenine + 1 mg/l BAP 200 mg/l claforan
A_{13} MS-salt/2	+ 3 % sucrose 0.5 MES g/l pH 5.7 0.7 % agar 200 mg/l claforan
Bacterial medium = min A :	(Miller 1972) 60 mM K_2HPO_4, $3H_2O$, 33 mM KH_2PO_4 ; 75 mM $(NH_4)_2SO4$ 1.7 M trinatriumcitrat; 1 mM $MgSO_4$; 2 g/l glucose ; 50 mg/l vitamine B1

- Plant material :
  Nicotiana tabacum cv. Petit Havana SR1
  Plants are used 6 to 8 weeks after subculture on medium A_1
- Infection :
  - midribs and edges are removed from leaves.
  - Remaining parts are cut into segments of about 0.25 cm^2 and are placed in the infection medium A_{10} (about 12 segments in a 9 cm Petri dish containing 10 ml A_{10}).
  - Segments are then infected with 25 µl per Petri dish of a late log culture of the Agrobacterium strain grown in min A medium.
  - Petri dish are incubated for 2 to 3 days at low light intensity.
  - After 2 to 3 days medium is removed and replaced by 20 ml of medium A_{10} containing 500 mg/l clarofan.
- Selection and shoot induction
  - The leaf discs are placed on medium A_{11} containing a selective agent :
    100 mg/l kanamycin and
    10 to 100 mg/l phosphinotricin.
  - Leaf discs are transferred to fresh medium weekly.

- After 3 to 4 weeks regenating calli arise. They are separated and placed on medium A_{12} with the same concentration of selective agent as used for the selection.

- Rooting
  - After 2 to 3 weeks the calli are covered with shoots, which can be isolated and transferred to rooting medium A_{13} (without selection).
  - Rooting takes 1 to 2 weeks.
  - After a few more weeks, these plants are propagated on medium A_1.

2. Tuber disc infection of Solanum tuberosum (potato)

Used media are described thereafter :

C_1 B5-medium	+ 250 mg/l NH_4NO_3
	300 mg/l $(CaCH_2PO_4)_2$
	0.5 g/l MES pH 5.7
	0.5 g/l polyvinylpyrrolidone (PVP)
	40 g/l mannitol (=0.22M)
	0.8% agar
	40 mg/l adenine
C_2 B5-medium	+ 250 mg/l NH_4NO_3
	400 mg/l glutamine
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	40 g/l mannitol
	40 mg/l adenine
	0.8 % agar
	+ 0.5 mg/l transzeatine
	0.1 mg/l IAA
	500 mg/l clarofan
C_5 MS salt/2	+ 3 % sucrose
	0.7 % agar
	pH 5.7
C_7 B5-medium	+ 250 mg/l NH_4NO_3
	400 mg/l glutamine
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	20 g/l mannitol
	20 g/l glucose
	40 mg/l adenine
	0.6 % agarose
	+ 0.5 mg/l transzeatine
	0.1 mg/l IAA
	500 mg/l clarofan
C_8 B5-medium	+ 250 mg/l NH_4NO_3
	400 mg/l glutamine
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	20 g/l mannitol
	20 g/l glucose
	40 mg/l adenine
	0.6 % agarose
	+ 200 mg/l clarofan
	1 mg/l transzeatine
C_9 B5-medium	+ 250 mg/l NH_4NO_3
	400 mg/l glutamine
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	20 g/l mannitol
	20 g/l glucose

40 mg/l adenine
0.6 % agarose
+ 1 mg/l transzeatine
0.01 mg/l Gibberellic acid A_3 (GA_3)
100 mg/l clarofan

C_{11} MS salt/2 + 6 % sucrose
0.7 % agar

Bacterial medium = min A : (Miller 1972 60 mM $K_2HPO_4.3H_2O$;
33 mM KH_2PO_4; 7.5 mM $(NH_4)_2SO_4$;
1.7 trinatriumcitrat; 1 mM $MgSO_4$;
2 g/l glucose; 50 mg/l vitamine B1.

- PLant material
  Tubers of Solanum tuberosum c.v. Berolina c.v. Désirée
- Infection
  - Potatoes are peeled and washed with water.
  - Then they are washed with concentrated commercial bleach for 20 minutes, and
  - rinsed 3 to 5 times with sterile water.
  - The outer layer is removed (1 to 1.5 cm)
  - The central part is cut into discs of about 1 cm^2 and 2 to 3 mm thick.
  - Discs are placed on medium C_1 (4 pieces in a 9 cm Petri dish).
  - 10 µl of a late log culture of an Agrobacterium strain grown in min A medium is applied on each disc.
  - Discs are incubated for 2 days at low light intensity.
- Selection and shoot induction
  - Discs are dried on a filter paper and transferred to medium C_2 with 100 mg/l kanamycin.
  - After one month small calli are removed from the discs and transferred to medium C_7 containing 50 mg/l kanamycin.
  - After a few more weeks, the calli are transferred to medium C_8 containing 50 mg/l kanamycin.
  - If little shoots start to develop, the calli are transferred to elongation medium C_9 containing 50 mg/l Kanamycin.
- Rooting
  - Elongated shoots are separated and transferred to rooting medium C_{11}.
  - Rooted shoots are propagated on medium C_5.

3. Leaf disc infection of Lycopersicum esculentum (tomato)

Used media are described thereafter

A_1 MS salt/2 + 1 % sucrose
0.8 % agar
pH 5.7

B_1 B5-medium + 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
0.5 g/l PVP
300 mg/l $Ca(H_2PO_4)_2$
2 % glucose
40 mg/l adenine
40 g/l mannitol

B_2 B5-medium + 250 mg/l NH_4NO_3
0.5 g/l MES pH 5.7
0.5 g/l PVP
400 mg/l glutamine
2 % glucose
0.6 % agarose
40 mg/l adenine
40 g/l mannitol
+ 0.5 mg/l transzeatine
0.01 mg/l IAA
500 mg/l claforan

B_3 B5-medium	+ 250 mg/l NH_4NO_3
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	400 mg/l glutamine
	2 % glucose
	0.6 % agarose
	40 mg/l adenine
	30 g/l mannitol
	+ 0.5 mg/l transzeatine
	0.01 mg/l IAA
	500 mg/l clarofan
B_4 B5-medium	+ 250 mg/l NH_4NO_3
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	400 mg/l glutamine
	2 % glucose
	0.6 % agarose
	40 mg/l adenine
	20 g/l mannitol
	+ 0.5 mg/l transzeatine
	0.01 mg/l IAA
	500 mg/l clarofan
B_5 B5-medium	+ 250 mg/l NH_4NO_3
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	400 mg/l glutamine
	2 % glucose
	0.6 % agarose
	40 mg/l adenine
	10 g/l mannitol
	+ 0.5 mg/l transzeatine
	0.01 mg/l IAA
	500 mg/l clarofan
B_6 B5-medium	+ 250 mg/l NH_4NO_3
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	400 mg/l glutamine
	2 % glucose
	0.6 % agarose
	40 mg/l adenine
	+ 0.5 mg/l transzeatine
	0.01 mg/l IAA
	200 mg/l clarofan
B_7 B5-medium	+ 250 mg/l NH_4NO_3
	0.5 g/l MES pH 5.7
	0.5 g/l PVP
	400 mg/l glutamine
	2 % glucose
	0.6 % agarose
	40 mg/l adenine
	+ 1 mg/l transzeatine
	200 mg/l clarofan
B_8 MS salt/2	+ 2 % sucrose
	0.5 g/l MES pH 5.7
	0.7 % agar
B_9 B5-medium	+ 250 mg/l NH_4NO_3
	0.5 g/l MES pH 5.7
	0.5 g/l PVP

2 % glucose
0.6 % agarose
40 mg/l adenine
+ 1 mg/l transzeatine
0.01 mg/l GA_3

Bacterial medium = min A : (Miller 1972) 60 mM $K_2HPO_4.3H_2O$;
33 mM KH_2PO_4; 7.5 mM $(NH_4)_2SO_4$;
1.7 M trinatriumcitrat; 1 mM $MgSO_4$;
2 g/l glucose; 50 mg/l vitamine B1

- Plant material
  Lycopersicum esculentum cv. Lucullus.
  Plants are used 6 weeks after subculture on medium A_1.
- Infection
  - Midrib is removed from the leaves.
  - Leaves are cut in segments of about 0.25 to 1 cm^2 (the edges of the leaves are not wounded, so that only maximum 3 sides of the leaf pieces is wounded).
  - Segments are placed in infection medium B_1 (upside down), about 10 segments in a 9 cm Petri dish.
  - Segments are then infected wiht 20 µl per Petri dish of a late log culture of the Agrobacterium strain grown in min A medium.
  - Petri dishes incubate for 2 days at low light intensity.
  - Medium is removed after 2 days and replaced by 20 ml of medium B_1 containing 500 mg/l clarofan.
- Selection and shoot induction
  - The leaf discs are placed in medium B_2 + 50 or 100 mg/l kanamycin.
  - Each 5 days the osmotic pressure of the medium is lowered by decreasing the mannitol concentration, transfers are done consecutively in medium B_3, B_4, B_5, and B_6.
  - After one month calli with meristems are separated from the leaf discs and placed on medium B_7 with 50 or 100 mg/l kanamycin.
  - Once little shoots have formed, calli are transferred to elongation medium B_9 with 50 or 100 mg/l kanamycin.
- Rooting
  - Elongated shoots are separated and transferred to medium B_8 for rooting.
  - Plants are propagated on medium A_1.

Greenhouse tests for herbicide resistance

Material and method

In this experiment, two herbicides comprising phosphinotricin as active ingredient, are used.

These compounds are those commercially available under the registered trademarks BASTA^R and MEIJI HERBIACE^R.

These products are diluted to 2 % with tap water. Spraying is carried out on a square metre area from the four corners. Temperature of the greenhouse is about 22 °C for tobaccos and tomato, and above 10 °C to 15 °C for potato.

Results

- Tobacco spraytest

a) Nicotiana tabacum cv. Petit Havana SR1 plants transformed with the chimeric "sfr" genes as present in pGSFR1161 or pGSFR1281, as well as unstransformed control plants (from 10 cm to 50 cm high) are treated with 20 l BASTA^R/ha. Control SR1 plants die after 6 days, while transformed plants are fully resistant to 20 l BASTA^R/ha and continue growing undistinguishable from untreated plants. No visible damage is detected, also the treatment is repeated every two weeks. The treatment has no effect in subsequent flowering. The recommended dose oF BASTA^R herbicide in agriculture is 2.5-7.5 l/ha.

b) A similar experiment is performed using 8 l/ha MEIJI HERBIACE^R. The transformed plants (the same as above) are fully resistant and continue growing undistinguishable from untreated plants. No

visible damage is detectable.

- Potato spraytest

  Untransformed and transformed potato plants (Solanum tuberosum cv. Berolina) (20 cm high) with the chimeric "sfr" gene as present in pGSFR1161 or pGSFR1281 are treated with 20 l BASTA^R/ha. Control plants die after 6 days while the transformed plants do not show any visible damage. They grow undistinguishable from untreated plants.

- tomato spraytest

  Untransformed and transformed tomato plants (lycopersium esculentum c.v. luculus) (25 cm high) with the chimeric "sfr" gene as present in pGSFR1161 and pGSFR1281 are treated with 20 l BASTA^R/ha. control plants die after six days while transformed plants are fully resistant. They do not show any visible damage and grow undistinguishable from untreated plants.

- Growth control of phytopathogenic fungi with transformed plants

  In another set of experiments, potato plants expressing chimeric "sfr" genes as present in pGSFR1161 or pGSFR1281 are grown in a greenhouse compartment at 20 °C under high humidity. Plants are innoculated by spraying 1 ml of a suspension of 10^5 Phytophtora infestans spores per ml. Plants grow in growth chambers (20 °c, 95 % humidity, 4 000 lux) until fungal disease symptoms are visible (one week). One set of the plants are at that moment sprayed with Bialaphos at a dose of 8 l/ha. Two weeks later, untreated plants are completely ingested by the fungus. The growth of the fungus is stopped on the Bialaphos treated plants and no further disease symptoms evolve. The plants are effectively protected by the fungicide action of Bialaphos.

- Transmission of the PPT resistance through seeds

  Transformed tobacco plants expressing the chimeric "sfr" gene present in pGSFR1161 and pGSFR1281 are brought to flowering in the greenhouse. They show a normal fertility.

About 500 F1 seeds of each plant are sown in soil, F1 designating seeds of the first generation, i.e directly issued from the originally transformed plants. When seedlings are 2-3 cm high, they are sprayed with 8 l BASTA^R/ha. 7 days later, healthy and damaged plants can be distinguished in a ratio of approximately 3 to 1. this shows that PPT resistance is inherited as a dominant marker encoded by a single locus.

10 resistant F1 seedlings are grown to maturity and seeds are harvested. F2 seedlings are grown as described above and tested for PPT-resistance by spraying BASTA^R at a dose of 8 l/ha. Some of the F1 plants produce F2 seedlings which are all PPT-resistant showing that these plants are homozygous for the resistance gene.The invention also concerns plant cells and plants non-essentially-biologically-transformed with a GS inhibitor-inactivating-gene according to the invention.

In a preferred embodiment of the invention, plant cells and plants are non-biologically-transformed with the "sfr" gene hereabove described.

Such plant cells and plants possess, stably integrated in their genome, a non-variety-specific character which render them able to produce detectable amounts of phosphinotricin-acetyl transferase.

This character confers to the transformed plant cells and plants a non-variety-specific enzymatic activity capable of inactivating or neutralizing GS inhibitors like Bialaphos and PPT.

Accordingly, plant cells and plants transformed according to the invention are rendered resistant against the herbicidal effects of Bialaphos and related compounds.

Since Bialaphos was first described as a fungicide, transformed plants can also be protected against fungal diseases by spraying with the compound several times.

In a preferred embodiment, Bialaphos or related compounds is applied several times, particularly at time intervals of about 20 to 100 days.

The invention also concerns a new process for selectively protecting a plant species against fungal diseases and selectively destroying weeds in a field comprising the steps of treating a field with an herbicide, wherein the plant species contain in their genome a DNA fragment encoding a protein having an enzymatic activity capable of neutralizing or inactivating GS inhibitors and wherein the used herbicide comprises as active ingredient a GS inhibitor.

It comes without saying that the process according to the invention can be employed with the same efficiency, either to only destroy weeds in a field, if plants are not infected with fungi , either to only stop the development of fungi if the latter appears after destruction of weeds.

In a preferred embodiment of the process according to the invention, plant species are transformed with a DNA fragment comprising the "sfr" gene as described hereabove, and the used herbicide is PPT or a related compound.

Accordingly, a solution of PPT or related compound is applied over the field, for example by spraying, several times after emergence of the plant species to be cultivated, until early and late germinating weeds

are destroyed.

It is quite evident that before emergence of plant species to be cultivated, the field can be treated with an herbicidal composition to destroy weeds.

On the same hand, fields can be treated even before the plant species to be cultivated are sowed.

Before emergence of the desired plant species, fields can be treated with any available herbicide, including Bialaphos-type herbicides.

After emergence of the desired plant species, Bialaphos or related compound is applied several times.

In a preferred embodiment, the herbicide is applied at time intervals of about from 20 to 100 days.

Since plants to be cultivated are transformed in such a way as to resist to the herbicidal effects of Bialaphos-type herbicides, fields can be treated even after emergence of the cultivated plants.

This is particularly useful to totally destroy early and late germinating weeds, without any effect on the plants to be produced.

Preferably, Bialaphos or related compoud is applied at a dose ranging from about 0.4 to about 1.6 kg/ha, and diluted in a liquid carrier at a concentration such as to enable its application to the field at a rate ranging from about 2 to about 8 l/ha.

There follows examples, given by way of illustration, of some embodiments of the process with different plant species.

- Sugarbeets

The North European sugarbeet is planted from March 15 up to April 15, depending upon the weather condition and more precisely on the precipitation and average temperature. the weeds problems are more or less the same in each country and can cause difficulties until the crop closes its canopy around mid-July.

Weed problems can be separated in three situations :

- early germination of the grassy weeds,
- early germinating broadleaved weeds,
- late germinating broadleaved weeds.

Up to now, pre-emergence herbicides have been successfully used. Such compounds are for example those commercially available under the registered trademarks : PYRAMIN^R, GOLTIX^R and VENZAR^R. However, the susceptibility to dry weather conditions of these products as well as the lack of residual activity to control late germinating weeds have led the farmer to use post-emergence products in addition to pre-emergence ones.

Table (I) thereafter indicates the active ingredients contained in the herbicidal compositions cited in the following examples.

TABLE (I)

Commercial Name	Active Ingredient	Formulation
AVADEX^R	Diallate	EC 400 g/l
AVADEX BW^R	Triallate	EC 400 g/l
GOLTIX^R	Metamitron	WP 70 %
RONEET^R	Cycloate	EC 718 g/l
TRAMAT^R	Ethofumerate	EC 200 g/l
FERVINAL^R	Alloxydime-sodium	SP 75 %
BASTA^R	Phosphinotricin	200 g/l
PYRAMIN FL^R	Chloridazon	SC 430 g/l

According to the invention, post-emergence herbicides consist of Bialaphos or related compounds, which offer a good level of growth control of annual grasses (Bromus, Avena spp., Alopecurus, POA) and broadleaves (Galium, Polygonum, Senecio, Solanum, Mercurialis).

Post-emergence herbicides can be applied at different moments of the growth of sugarbeet ; at a

cotyledon level, two-leave level or at a four-leave level.

Table (II) thereafter represents possible systems of field-treatment, given by way of example.

In those examples, the post-emergence herbicide of the class of Bialaphos used is BASTA^{R}, in combination with different pre-emergence herbicides. Concentrations are indicated in l/ha or kg/ha.

TABLE (II)

POSSIBLE HERBICIDAL SYSTEMS IN SUGARBEETS, BASED ON THE USE OF BASTA^{R} , PROVIDING BEETS ARE MADE RESISTANT AGAINST THE LATTER CHEMICAL (in lt or kg/ha).

	Pre-sowing	Pre-emergence	Cotyledons	Two-leaves	Four leaves
1.	AVADEX^{R} 3.5 lt	-	BASTA^{R} 3 lt	BASTA^{R}/tramat 3 lt 1.5 lt	-
2.	AVADEX^{R} 3.5 lt	GOLTIX^{R} 4 kg	-	-	-
3	RONEET^{R} 4 lt	GOLTIX^{R} 5 kg	-	-	-
4.	RONEET^{R} 4 lt	GOLTIX^{R} 2.5 kg	-	BASTA^{R} 3 lt	-
5.	TRAMAT^{R} 5 lt	-	-	BASTA^{R} 3 lt	BASTA^{R}/GOLTIX^{R} 2 lt 2 kg
6.	-	GOLTIX^{R} 2.5 kg	-	BASTA^{R} 3 lt	-
7.	-	-	BASTA^{R}/tramat 3 lt 1.7 lt	-	BASTA^{R}/GOLTIX^{R} 3 lt 2 kg
8.	PYRAMIN^{R} 6 lt	-	BASTA^{R} 3 lt	Venzar 1 kg	-
9.	-	-	BASTA^{R} 3 lt	BASTA^{R}/GOLTIX^{R} 3 lt 2 kg	-
10.	DIALLATE^{R} 3.5 lt	PYRAMIN^{R} 6 lt		BASTA^{R}/Metamitron 3 lt 1 kg	-

- Potatoes

Potatoes are grown in Europe on about 8.10^{6} Ha. The major products used for weed control are

Linuron/monolinuron or the compound commercially available under the denomination METRABUZIN

These products perform well against most weedspecies.

However, weeds such as Galium and Solanum plus late germinating Chenopodium and Polygonum are not always effectively controlled, while control of the annual grasses is also sometime erratic.

Once again, late germinating broadleaved weeds are only controllable by post-emergence applications of herbicides such as BASTA^R.

Table (III) thereafter represents some examples given by way of example of field-treatment in the case of potatoes.

TABLE (III)

Weeds control systems in potatoes, based on the use of BASTA^R, providing potatoes are rendered resistant to BASTA^R.

Linuron + monolinuron (375 g + 375 g/ha) prior to emergence	
BASTA^R	**3-4 lt/ha after emergence (5-15 cm)**
BASTA^R/fluazifop-butyl	**3-4 lt/ha + 2 lt/ha after emergence (5-15 cm)**
Linuron	**WP 50 % (AFALON^R)**
Monolinuron	**WP 47.5 % (ARESSIN^R)**
fluazifop-butyl	**EL 250 g/l (FUSILADE^R)**

The strains pGSJ260 and pBG39 used hereabove have been deposited on December 12nd, 1985, at the "German Collection of Micro-organisms" (DEUTSCHE SAMMLUNG VON MIKROORGANISMEN) at Göttingen, Germany. They received the deposition numbers DSM 3 606 and DSM 3 607 respectively.

There follows hereafter the description of experiments carried out for the isolation of the "sfrsv" resistance gene, the construction of expression vectors which contain the resistance gene and which allow the subsequent transformation of plant cells, in order to render them resistant to GS inhibitors.

Cloning of the bialaphos-resistance-"sfrsv" gene from Streptomyces viridochromogenes

The strain Streptomyces viridochromogenes DSM 40736 (ref 1) was grown and total DNA of this strain was prepared according to standard techniques. DNA samples were digested respectively with PstI, SmaI and Sau3AI in three different reactions and separated on an agarose gel, together with plasmid DNA from pGSR1 (fig. 5B) digested with BamHI. In a Southern analysis the DNA was blotted on a nitrocellulose filter and hybridized with the labbeled BamHI fragment from pGSR1 containing the "sfr" gene. In all four lanes of the gel, a restriction fragment was showing strong homology with the probe : a PstI fragment of about 3 kb, a SmaI fragment of about 1.2 kb and Sau3AI fragment of 0.5 kb. In order to clone this gene, PstI restriction fragments were directly cloned in the Escherichia coli vector pUC8. 3000 colonies obtained after transformation were transferred to nitrocellulose filters, and hybridized with the "sfr" probe. Positive candidates were further tested for their growth on minimal medium plates containing 300 µg/ml PPT. One transformant that grew on PPT-containing-medium was further analysed. The plasmid map and relevant restriction sites of this plasmid pJS1 are represented in fig. 8. The strain MC1061 (pJS1) has been deposited on March 06,

1987 at the DEUTSCHE SAMMLUNG VON MIKROORGANISMEN (DSM) under deposition number DSM 4023. The clone restriction fragment has been sequenced according to the Maxam and Gilbert method and the coding region of the gene could be identified through homology. The sequence of the "sfrsv" gene is represented in fig.9 and the homology on the nucleotide and amino acid sequence level with "sfr" gene is shown in fig. 10.

Expression of the "sfrsv" gene

A "sfrsv gene cassette" was also constructed to allow subsequent cloning in plant expression vectors. A BanII-BglII fragment containing the "sfrsv" coding region without the initiation codon GTG was isolated from pJS1. This fragment was ligated in the vector pLK56-2 digested with NcoI and BglII, together with a synthetic oligonucleotide 5'-CATGAGCC-3', similar with the one described for "sfr" gene and shown in fig. 5. The construction of pGSR1SV is schematically shown in fig. 11. Since similar cassettes of both genes are present in respectively pGSR1 and pGSR1SV, previous described constructions for the expression of the "sfr" gene in plants can be repeated.

Enzymatic analysis of crude extracts from E. coli strains carrying plasmid pGSR1SV demonstrated the synthesis of an acetylase which could acetylate PPT. This was shown by thin layer chromotography of the reaction porducts.

The "sfrsv" gene was then inserted into the plasmid vector pGSJ260 (fig. 4B) under the control of the CaMV 35s promoter, to yield a plasmid pGS2SV, similar to pGSR2 (fig. 6A) except that the "sfrsv" gene is substituted for the "sfr" gene.

It is clear that herbicide resistance genes of the above type may be obtained from many other microorganisms that produce PPT or PPT derivatives. Herbicide resistance gene can then be incorporated in plant cells with a view of protecting them against the action of such Glutamine Synthetase inhibitors. For instance, a Bialaphos-resistance-gene is obtained from Kitasotosporia (ref. 15).

Transformed plant cells and plants which contain the "sfrsv" resistance gene can be obtained with plasmid pGSR2SV, using the same Agrobacterium-mediated-transformation system as hereabove described for the transformation of different plant species with the "sfr" gene.

Plants are regenerated and tested for their resistance with similar spraying tests as described hereabove. All plants behaved similarly and show resistance against herbicides consisting of glutamine synthetase inhibitors.

Finally, the inventors also pertains to the combination of the plants resistant to an inhibitor of glutamine synthetase as defined above with the corresponding inhibitor of glutamine synthetase for use in the production of cultures of said plants free form weeds.

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Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. A DNA fragment, for the subsequent transformation of plant cells, coding for a polypeptide having phosphinotricin-acetyl-transferase activity, which consists of a nucleotide sequence coding for the following amino acid sequence :

V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N F

R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

H L L K S M E A Q G F K S V V A V I

G L P N D P S V R L H E A L G Y T A

R G T L R A A G Y K H G G W H D V G

F W Q R D F E L P A P P R P V R P V

T Q I *

or of a part of said nucleotide sequence of sufficient length to code for a polypeptide still having phosphinotricin-acetyl-transferase activity.

2. The DNA fragment of claim 1, which comprises the following nucleotide sequence :

or of a part thereof expressing a polypeptide having phosphinotricin-acetyl-transferase activity.

3. The fragment of claim 2 wherein the initiation codon ATG is substituted for the initiation CTG.

4. Process for controlling the action in plant cells and plants comprising such cells of a glutamine synthetase inhibitor when the former are contacted with the latter, which comprises causing the stable integration in the genomic DNA of said plant cells of a heterologous DNA including a promoter recognized by plymerases of said plant cells and a foreign nucleotide sequence capable of being expressed in the form of a protein in said plant cells, and wherein said protein has an enzymatic activity capable of causing inactivation or neutralization of said glutamine synthetase inhibitor, characterized in that said foreign nucleotide sequence is the nucleotide sequence or the DNA fragment of any of claims 1 to 3.

5. Process for producing a plant or reproduction material of said plant including a heterologous genetic material stably integrated therein and capable of being expressed in said plants or reproduction material in the form of a protein capable of inactivating or neutralizing the activity of a glutamine synthetase inhibitor, which process comprises transforming cells or tissue of said plants with a DNA recombinant containing a heterologous DNA, as well as the regulatory elements selected among those which are capable of causing the stable integration of said heterologous DNA in said plant cells or

tissue and of enabling the expression of said foreign nucleotide sequence in said plant cells or plant tissue,
regenerating plants or reproduction material of said plants or both from the plants cells or tissue transformed with said heterologous DNA and, optionally, biologically replicating said last mentioned plants or reproduction material or both,
characterized in that said heterologous DNA has the nucleotide sequence of the DNA fragment of any of claims 1 to 3 or of said part that codes for a protein having phosphinotricinacetyl transferase activity.

6. The process according to claim 5, wherein the recombinant DNA is a vector suitable for the transformation of the cells of said plant.

7. The process of claim 5 or 6, wherein the vector comprises a nucleotide sequence encoding a transit peptide intercalated between said plant promotor region and said recombinant DNA fragment.

8. The process of claim 7, wherein the transit peptide is selected from ribulose-1,5 biphosphate carboxylase and chlorophyl a/b binding proteins.

9. The process of any of claims 6 to 8, wherein said vector is a Ti plasmid.

10. Plant cells, non-essentially biologically transformed, which possess, stably integrated in their genome, the heterologous DNA of any of claims 1 to 3.

11. Plant cells according to claim 10, which can be regenerated into a plant capable of producing seeds.

12. Plant cells according to any of claims 10 and 11, which produce detectable amounts of phospinotricin acetyl transferase.

13. Seeds, which possess, stably integrated in their genome, a DNA fragment as defined in any of claims 1 to 3.

14. Seeds according to claim 12, which are capable of germinating into a plant capable of producing seeds having a non-variety-specific enzymatic activity capable of inactivating or neutralizing glutamine synthetase inhibitors.

15. Seeds according to claim 13 or 14, which are transformed by the process of any one of the claims 4 to 8.

16. Plants, non-essentially biologically transformed, which possess, stably integrated in the genome of their cells, a heterologous DNA as defined in any of claims 1 to 3.

17. Plants according to claim 16, which are capable of producing seeds.

18. Plants according to claim 16 or 17, which are transformed by the process of claim 5.

19. Process for selectively protecting a plant species and selectively destroying weeds in a fiels which comprises the steps of treating a field with an herbicide, wherein the plant species contain in their genome a heterologous DNA as defined in any of claims 1 to 3, and wherein the used herbicide is a glutamine synthetase inhibitor.

20. Process according to claim 18 or 19, wherein the plant species are transformed according to the process of any one of claims 4 to 8.

21. Process according to claim 18 or 19, wherein a solution of a glutamine synthetase inhibitor is applied on the field, after emergence of the cultivated plant species, several times, particularly at time intervals of about 20 to 100 days, until early and late germinating weeds are destroyed.

22. Process according to claim 18 or 19, wherein glutamine synthetase inhibitors comprise Bialaphos, phosphinotricin and related compounds.

23. Process for selectively protecting a plant species in a field against fungal diseases comprising the steps of treating a field with a herbicide consisting of a glutamine synthetase inhibitor, wherein the plant species contain in the genome of its cells a heterologous DNA as defined in any of claims 1 to 3 and wherein the used herbicide is a glutamine synthetase inhibitor.

24. Process according to claim 23, wherein the plant species are transformed according to the process of claim 5.

25. Process according to claims 23 or 24, wherein a solution of a glutamine synthetase inhibitor is applied on the field, after emergence of the cultivated plant species, several times, particularly at time intervals of about 20 to 100 days until fungi are destroyed.

26. Process according to any one of claims 23 to 25, wherein glutamine synthetase inhibitor is selected from a group which comprises Bialaphos, phosphinotricin and related compounds.

27. Process according to claim 22 or 26, wherein Bialaphos, PPT or the related compound is applied at a dose ranging from about 0.4 to about 1.6 kg/ha.

28. Process according to claim 27, wherein Bialaphos, PPT or the related compound is diluted in a liquid carrier at a concentration such as to enable its application to the field at a rate ranging from about 2 l/ha to about 8 l/ha.

29. Process according to claim 27 or 28, wherein selectively protected plant species comprise sugarbeet, rice, potato, tomato, maize, tobacco.

30. Vector which contains a DNA fragment as defined in any of claims 1 to 3.

31. Vector according to claim 30, which is suitable for the transformation of plant cells and plants.

32. The vector of claim 31, wherein said DNA fragment is under the control of a plant promoter region and of regulation elements allowing for the expression of said DNA fragments in plant cells, when the latter are later transformed with said vector.

33. The vector of claim 32, which confers bialaphos-resistance to the transformed plant cells.

34. The vector of claim 33, wherein a sequence encoding a transit peptide is intercalated between said plant promoter region and said DNA fragment.

35. The vector of claim 34, wherein the transit peptide is selected from ribulose-1,5 biphosphate carboxylase and chlorophyl a/b binding proteins.

36. The vector of any of claims 31 to 35, wherein said vector is a modified Ti plasmid.

37. The vector of claim 29, wherein said DNA fragment is under the control of replicon elements suitable for the transformation of bacteria, particularly E. coli.

Claims for the following Contracting States : AT, ES

1. A process, for the transformation of plant cells, which comprises transforming said plant cells with a vector suitable for the transformation of plant cells and which contains a DNA sequence coding for a polypeptide having phosphinotricin-acetyl-transferase activity, which consists of a nucleotide sequence coding for the following amino acid sequence :

V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N P

R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

H L L K S M E A Q G F K S V V A V I

G L P N D P S V R L H E A L G Y T A

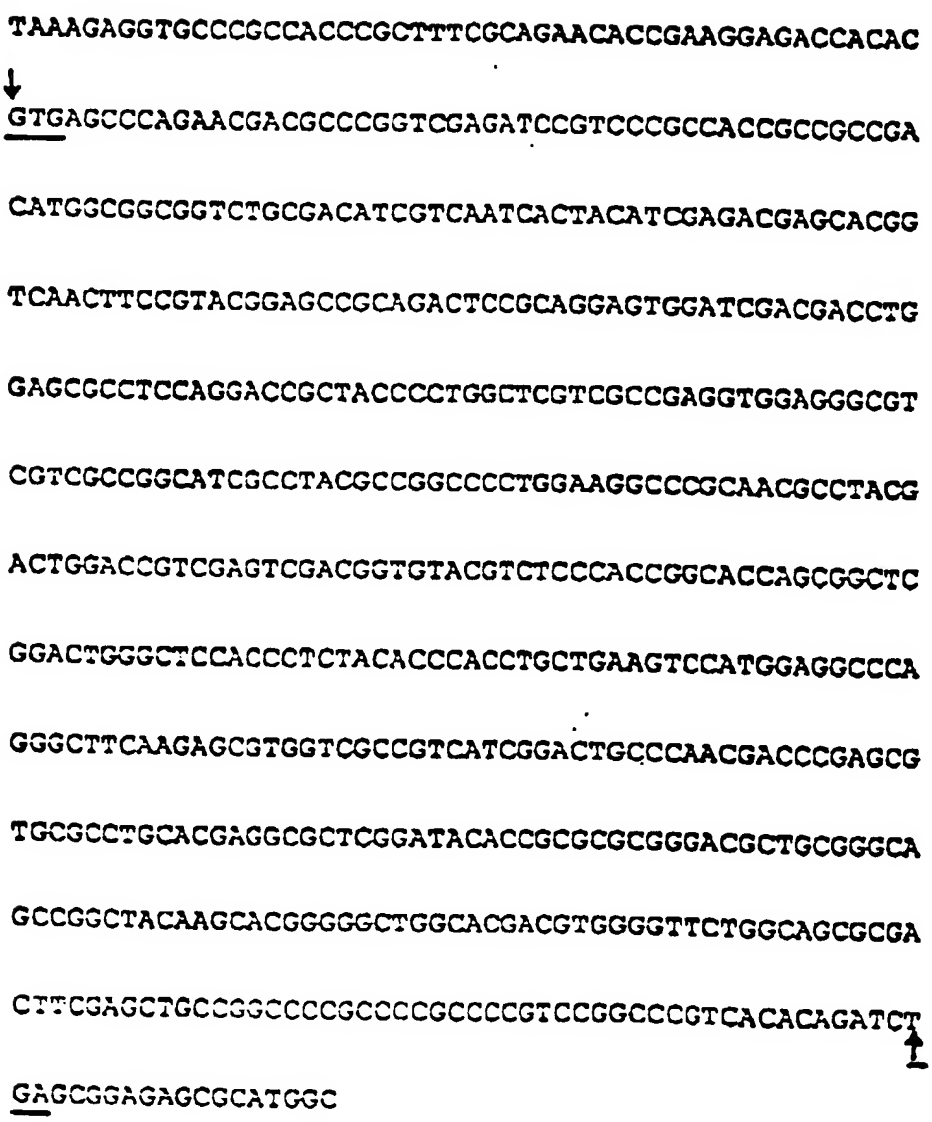
R G T L R A A G Y K H G G W H D V G

F W Q R D F E L P A P P R P V R P V

T Q I *

or of a part of said nucleotide sequence of sufficient length to code for a polypeptide still having phosphinotricin-acetyl-transferase activity.

2. The process of claim 1, in which said DNA sequence comprises the following nucleotide sequence :



or of a part thereof expressing a polypeptide having phosphinotricin-acetyl-transferase activity.

3. The process of claim 2 wherein the initiation codon ATG is substituted for the initiation CTG of said nucleotide sequence.

4. Process for controlling the action in plant cells and plants comprising such cells of a glutamine synthetase inhibitor when the former are contacted with the latter, which comprises causing the stable integration in the genomic DNA of said plant cells of a heterologous DNA including a promoter recognized by polymerases of said plant cells and a foreign nucleotide sequence capable of being expressed in the form of a protein in said plant cells, and wherein said protein has an enzymatic activity capable of causing inactivation or neutralization of said glutamine synthetase inhibitor, characterized in that said foreign nucleotide sequence is the nucleotide sequence defined in any of claims 1 to 3 or part thereof.

5. Process for producing a plant or reproduction material of said plant including a heterologous genetic material stably integrated therein and capable of being expressed in said plants or reproduction material in the form of a protein capable of inactivating or neutralizing the activity of a glutamine

synthetase inhibitor, which process comprises transforming cells or tissue of said plants with a DNA recombinant containing the heterologous DNA of claim 4, as well as the regulatory elements selected among those which are capable of causing the stable integration of said heterologous DNA in said plant cells or tissue and of enabling the expression of said foreign nucleotide sequence in said plant cells or plant tissue,
regenerating plants or reproduction material of said plants or both from the plant cells or tissue transformed with said heterologous DNA and, optionally, biologically replicating said last mentioned plants or reproduction material or both,
characterized in that said heterologous DNA has the nucleotide sequence defined in any of claims 1 to 3 or of said part that codes for a polypeptide having phosphinotricin-acetyl transferase activity.

6. The process according to claim 5, wherein the recombinant DNA is a vector suitable for the transformation of the cells of said plant.

7. The process of claim 5 or 6, wherein the vector comprises a nucleotide sequence encoding a transit peptide intercalated between said plant promotor region and said recombinant DNA fragment.

8. The process of claim 7, wherein the transit peptide is selected from ribulose-1,5 biphosphate carboxylase and chlorophyl a/b binding proteins.

9. The process of any of claims 6 to 8, wherein said vector is a Ti plasmid.

10. Plant cells, non-essentially biologically transformed, which possess, stably integrated in their genome, the heterologous sequence defined in any of claims 1 to 3.

11. Plant cells according to claim 10, which can be re-generated into a plant capable of producing seeds.

12. Plant cells according to any of claims 10 and 11, which produce detectable amounts of phospinotricin acetyl transferase.

13. Seeds, which possess, stably integrated in their genome, a DNA sequence as defined in any of claims 1 to 3.

14. Seeds according to claim 12, which are capable of germinating into a plant capable of producing seeds having a non-variety-specific enzymatic activity capable of inactivating or neutralizing glutamine synthetase inhibitors.

15. Seeds according to claim 13 or 14, which are transformed by the process of any one of the claims 4 to 8.

16. Plants, non-essentially biologically transformed, which possess, stably integrated in the genome of their cells, a heterologous DNA as defined in any of claims 1 to 3.

17. Plants according to claim 16, which are capable of producing seeds.

18. Plants according to claim 16 or 17, which are transformed by the process of claim 5.

19. Process for selectively protecting a plant species and selectively destroying weeds in a field which comprises the steps of treating a field with an herbicide wherein the plant species contain in their genome a heterologous DNA as defined in any of claims 1 to 3, and wherein the used herbicide is a glutamine synthetase inhibitor.

20. Process according to claim 18 or 19, wherein the plant species are transformed according to the process of any one of claims 4 to 8.

21. Process according to claim 18 or 19, wherein a solution of a glutamine synthetase inhibitor is applied on the field, after emergence of the cultivated plant species, several times, particularly at time intervals of about 20 to 100 days, until early and late germinating weeds are destroyed.

22. Process according to claim 18 or 19, wherein glutamine synthetase inhibitors comprise Bialaphos, phosphinotricin and related compounds.

23. Process for selectively protecting a plant species in a field against fungal diseases comprising the steps of treating a field with a herbicide consisting of a glutamine synthetase inhibitor, wherein the plant species contain in the genome its cells a heterologous DNA as defined in any of claims 1 to 3 and wherein the used herbicide is a glutamine synthetase inhibitor.

24. Process according to claim 23, wherein the plant species are transformed according to the process of claim 5.

25. Process according to claims 23 or 24, wherein a solution of a glutamine synthetase inhibitor is applied on the field, after emergence of the cultivated plant species, several times, particularly at time intervals of about 20 to 100 days until fungi are destroyed.

26. Process according to any one of claims 23 to 25, wherein glutamine synthetase inhibitor is selected from a group which comprises Bialaphos, phosphinotricin and related compounds.

27. Process according to claim 22 or 26, wherein Bialaphos, PPT or related compound is applied at a dose ranging from about 0.4 to about 1.6 kg/ha.

28. Process according to claim 27, wherein Bialaphos, PPT or related compound is diluted in a liquid carrier at a concentration such as to enable its application to the field at a rate ranging from about 2 l/ha to about 8 l/ha.

29. Process according to claim 27 or 28, wherein selectively protected plant species comprise sugarbeet, rice, potato, tomato, maize, tobacco.

30. A process for producing a vector which contains a DNA sequence as defined in any of claims 1 to 3 and which is suitable for the transformation of plant cells and plants, which comprises bringing said DNA sequence under the control of a plant promoter region, and of regulation elements contained in said vector and which allow for the expression of said DNA sequence in plant cells, when the latter are later transformed with said vector.

31. The process of claim 30, wherein said DNA sequence confers bialaphos-resistance to plant cells transformed with said vector.

32. The process of claim 31, wherein a sequence encoding a transit peptide is intercalated between said plant promoter region and said DNA fragment.

33. The process of claim 32, wherein the transit peptide is selected from ribulose-1,5 biphosphate carboxylase and chlorophyl a/b binding proteins.

34. The process of any of claims 30 to 33, wherein said vector is a modified Ti plasmid.

35. The process of claim 30, wherein said DNA sequence is under the control of replicon elements suitable for the transformation of bacteria, particularly E. coli.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. DNS-Fragment, für die anschließende Transformation von Pflanzenzellen, das für ein Polypeptid mit Phosphinotricinacetyl-transferase-Aktivität codiert, das aus einer Nucleotidsequenz besteht, die für die folgende Aminosäuresequenz codiert:

V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N F

R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

H L L K S M E A Q G F K S V V A V I

G L P N D P S V R L H E A L G Y T A

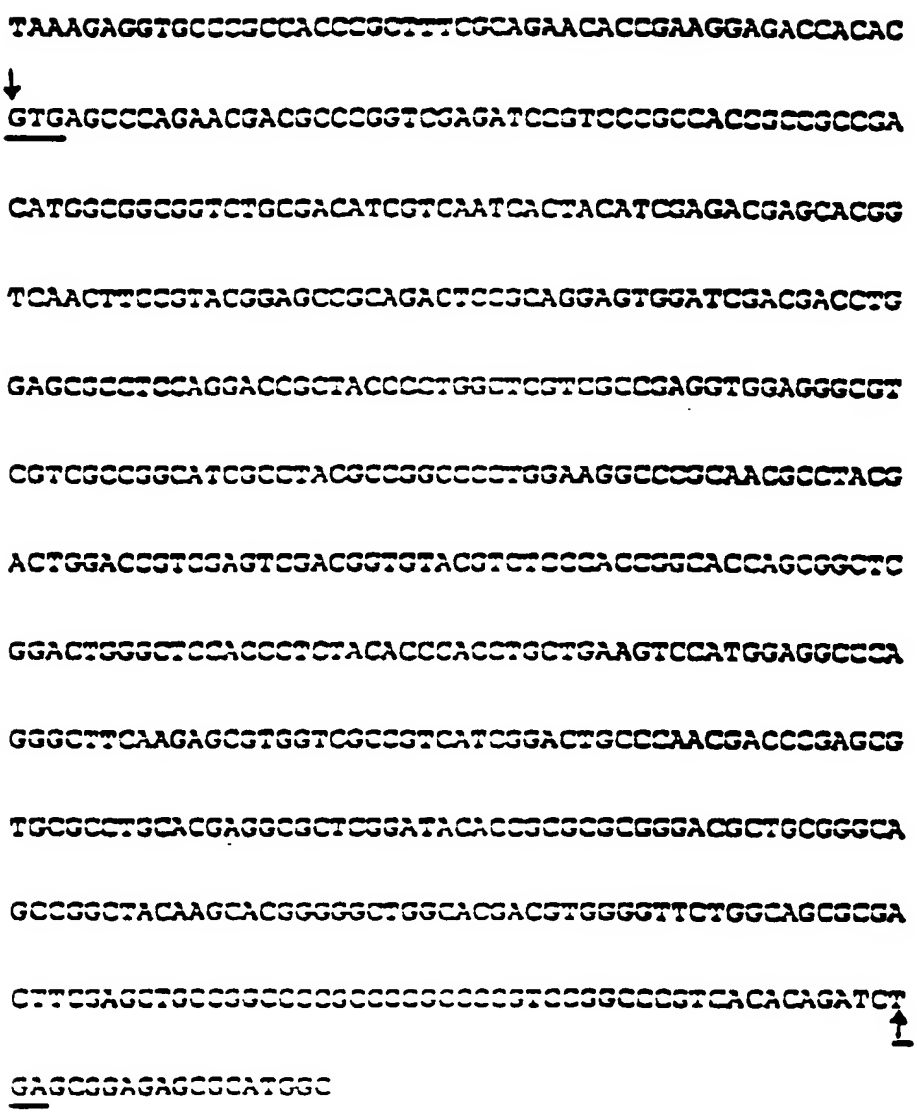
R G T L R A A G Y K H G G W H D V G

F W Q R D F E L P A P P R P V R P V

T Q I *

oder aus einem Teil der genannten Nucleotidsequenz von ausreichender Länge, um für ein Polypeptid zu codieren, das noch immer Phosphinotricin-acetyl-transferase-Aktivität besitzt.

2. DNS-Fragment nach Anspruch 1, das die folgende Nucleotidsequenz umfaßt:



oder einen Teil davon, der ein Polypeptid mit Phosphinotricin-acetyl-transferase-AKtivität exprimiert.

3. Fragment nach Anspruch 2, worin das Initiationscodon ATG eingesetzt ist für die Initiation CTG.

4. Verfahren zum Kontrollieren der Vorgänge in Pflanzenzellen und Pflanzen, die solche Zellen eines Glutaminsynthetaseinhibitors umfassen, wenn die ersteren mit dem letzteren kontaktiert werden, das das Bewirken der stabilen Integration in die genomische DNS der besagten Pflanzenzellen einer heterologen DNS umfaßt, einschließlich eines Promotors, der von Polymerasen der besagten Pflanzenzellen erkannt wird, und einer fremden Nucleotidsequenz, die in Form eines Proteins in den genannten Pflanzenzellen exprimiert werden kann, und worin das genannte Protein eine enzymatische Aktivität hat, die die Inaktivierung oder Neutralisation des genannten Glutaminsynthetaseinhibitors verursachen kann, dadurch gekennzeichnet, daß die besagte fremde Nucleotidsequenz die Nucleotidsequenz oder das DNS-Fragment aus einem der Ansprüche 1 bis 3 ist.

5. Verfahren zur Herstellung einer Pflanze oder von Reproduktionsmaterial dieser Pflanze, die ein heterologes genetisches Material stabil darin eingebaut umfaßt und in besagten Pflanzen oder Reproduktionsmaterial in der Form eines Proteins exprimiert werden kann, das die Aktivität eines Glutaminsynthetaseinhibitors inaktivieren oder neutralisieren kann, wobei dieses Verfahren das Transformieren

von Zellen oder Gewebe der besagten Pflanzen mit einer DNS-Rekombinante umfaßt, die sowohl heterologe DNS, als auch die Regulationselemente enthält, ausgewählt aus jenen, die die stabile Integration besagter heterologer DNS in besagten Pflanzenzellen oder Gewebe verursachen und die Expression der besagten fremden Nucleotidsequenz in besagten Pflanzenzellen oder Pflanzengewebe ermöglichen können,
Regenerieren von Pflanzen oder Reproduktionsmaterial besagter Pflanzen oder beides aus Pflanzenzellen oder Gewebe, das mit besagter heterologer DNS transformiert wurde und, fakultativ, biologische Replikation besagter zuletzt erwähnter Pflanzen oder von Reproduktionsmaterial oder von beiden, dadurch gekennzeichnet, daß besagte heterologe DNS die Nucleotidsequenz von dem DNS-Fragment aus einem der Ansprüche 1 bis 3 oder des genannten Teils der für ein Polypeptid mit Phosphinotricin-acetyl-transferase-Aktivität codiert.

6. Verfahren nach Anspruch 5, worin die rekombinante DNS ein Vektor geeignet zur Transformation der Zellen besagter Pflanzen ist.

7. Verfahren nach Anspruch 5 oder 6, worin der Vektor eine Nucleotidsequenz umfaßt, die für ein Transitpeptid codiert, eingeschoben zwischen besagtem Pflanzenpromotor-Bereich und besagtem rekombinanten DNS-Fragment.

8. Verfahren nach Anspruch 7, worin das Transitpeptid aus Ribulose-1,5-biphosphatcarboxylase und Chlorophyll-a/b-Bindungsproteinen ausgewählt ist.

9. Verfahren nach einem der Ansprüche 6 bis 8, worin besagter Vektor ein Ti-Plasmid ist.

10. Pflanzenzellen, nicht im wesentlichen biologisch transformiert, die, stabil integriert in ihr Genom, die heterologe DNS aus einem der Ansprüche 1 bis 3 besitzen.

11. Pflanzenzellen nach Anspruch 10, die zu einer Pflanze, die Samen produzieren kann, regeneriert werden können.

12. Pflanzenzellen nach einem der Ansprüche 10 oder 11, die nachweisbare Mengen Phosphinotricin-acetyl-transferase produzieren.

13. Samen, die, stabil integriert in ihr Genom, ein DNS-Fragment, wie in einem der Ansprüche 1 bis 3 definiert, besitzen.

14. Samen nach Anspruch 12, die zu einer Pflanze keimen können, die Samen produzieren kann, die eine nicht-sorten spezifische enzymatische Aktivität haben, die Glutaminsynthetaseinhibitoren inaktivieren oder neutralisieren kann.

15. Samen nach einem der Ansprüche 13 oder 14, die durch das Verfahren nach einem der Ansprüche 4 bis 8 transformiert wurden.

16. Pflanzen, nicht im wesentlichen biologisch transformiert, die, stabil integriert in das Genom ihrer Zellen, eine heterologe DNS, wie in einem der Ansprüche 1 bis 3 definiert, besitzen.

17. Pflanzen nach Anspruch 16, die Samen produzieren können.

18. Pflanzen nach Anspruch 16 oder 17, die durch das Verfahren nach Anspruch 5 transformiert wurden.

19. Verfahren zum selektiven Schützen einer Pflanzenart und selektiven Vernichten von Unkraut auf einem Feld, das die Schritte des Behandelns des Feldes mit einem Herbizid umfaßt, worin die Pflanzenart in ihrem Genom eine heterologe DNS, wie in einem der Ansprüche 1 bis 3 definiert, enthält und worin das verwendete Herbizid ein Glutaminsynthetaseinhibitor ist.

20. Verfahren nach Anspruch 18 oder 19, worin die Pflanzenarten gemäß dem Verfahren nach einem der Ansprüche 4 bis 8 transformiert werden.

21. Verfahren nach Anspruch 18 oder 19, worin eine Lösung eines Glutaminsynthetaseinhibitors auf das Feld nach dem Auswuchs der angebauten Pflanzenart mehrere Male aufgetragen wird, insbesondere nach Zeitabschnitten von etwa 20 bis 100 Tagen, bis früh- und spätkeimendes Unkraut vernichtet ist.

22. Verfahren nach Anspruch 18 oder 19, worin die Glutaminsynthetaseinhibitoren Bialaphos, Phosphinotricin und verwandte Verbindungen umfassen.

23. Verfahren zum selektiven Schutz einer Pflanzenart gegen Pilzkrankheiten auf einem Feld, das die Schritte des Behandelns eines Feldes mit einem Herbizid umfaßt, das aus einem Glutaminsynthetaseinhibitor besteht, worin die Pflanzenarten in dem Genom ihrer Zellen eine heterologe DNS, wie in einem der Ansprüche 1 bis 3 definiert, enthalten und worin das verwendete Herbizid ein Glutaminsynthetaseinhibitor ist.

24. Verfahren nach Anspruch 23, worin die Pflanzenarten gemäß dem Verfahren nach Anspruch 5 transformiert werden.

25. Verfahren nach Anspruch 23 oder 24, worin eine Lösung eines Glutaminsynthetaseinhibitors nach dem Auswuchs der angebauten Pflanzenarten mehrere Male auf das Feld aufgetragen wird, insbesondere nach Zeitabschnitten von etwa 20 bis 100 Tagen, bis die Pilze vernichtet sind.

26. Verfahren nach einem der Ansprüche 23 bis 25, worin der Glutaminsynthetaseinhibitor aus einer Gruppe, die Bialaphos, Phosphinotricin und verwandte Verbindungen umfaßt, ausgewählt ist.

27. Verfahren nach Anspruch 22 oder 26, worin Bialaphos, PPT oder die verwandte Verbindung in einer Dosis im Bereich von etwa 0,4 bis etwa 1,6 kg/ha aufgetragen wird.

28. Verfahren nach Anspruch 27, worin Bialaphos, PPT oder die verwandte Verbindung in einem flüssigen Träger auf eine Konzentration verdünnt wird, die ihre Auftragung auf das Feld bei einer Menge im Bereich von etwa 2 l/ha bis etwa 8 l/ha ermöglicht.

29. Verfahren nach Anspruch 27 oder 28, worin die selektiv geschützten Pflanzenarten Zuckerrübe, Reis, Kartoffel, Tomate, Mais und Tabak umfassen.

30. Vektor, der ein DNS-Fragment, wie in einem der Ansprüche 1 bis 3 definiert, enthält.

31. Vektor nach Anspruch 30, der für die Transformation von Pflanzenzellen und Pflanzen geeignet ist.

32. Vektor nach Anspruch 31, worin besagtes DNS-Fragment unter der Kontrolle eines Pflanzenpromotor-Bereichs und von Regulierungselementen ist, die die Expression von besagten DNS-Fragmenten in Pflanzenzellen, wenn letztere später mit besagtem Vektor transformiert werden, erlauben.

33. Vektor nach Anspruch 32, der Bialaphosresistenz den transformierten Pflanzenzellen verleiht.

34. Vektor nach Anspruch 33, worin eine Sequenz, die für ein Transitpeptid codiert, zwischen besagtem Pflanzenpromotor-Bereich und besagtem DNS-Fragment eingeschoben ist.

35. Vektor nach Anspruch 34, worin das Transitpeptid aus Ribulose-1,5-biphosphatcarboxylase und Chlorophyll-a/b-Bindungsproteinen ausgewählt ist.

36. Vektor nach einem der Ansprüche 31 bis 35, worin besagter Vektor ein modifiziertes Ti-Plasmid ist.

37. Vektor nach Anspruch 29, worin besagtes DNS-Fragment unter der Kontrolle von Replikationselementen ist, die für die Transformation von Bakterien, insbesondere E. coli, geeignet sind.

Patentansprüche für folgende Vertragsstaaten : AT, ES

1. Verfahren zur Transformation von Pflanzenzellen, das das Transformieren der besagten Pflanzenzellen mit einem, für die Transformation von Pflanzenzellen geeigneten Vektor umfaßt und der eine DNS-

Sequenz, die für ein Polypeptid codiert, enthält, das Phosphinotricin-acetyl-transferase-Aktivität besitzt, und die aus einer Nucleotidsequenz besteht, die für die folgende Aminosäuresequenz codiert:

V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N P

R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

H L L K S M E A Q G F K S V V A V I

C L P N D P S V R L H E A L G Y T A

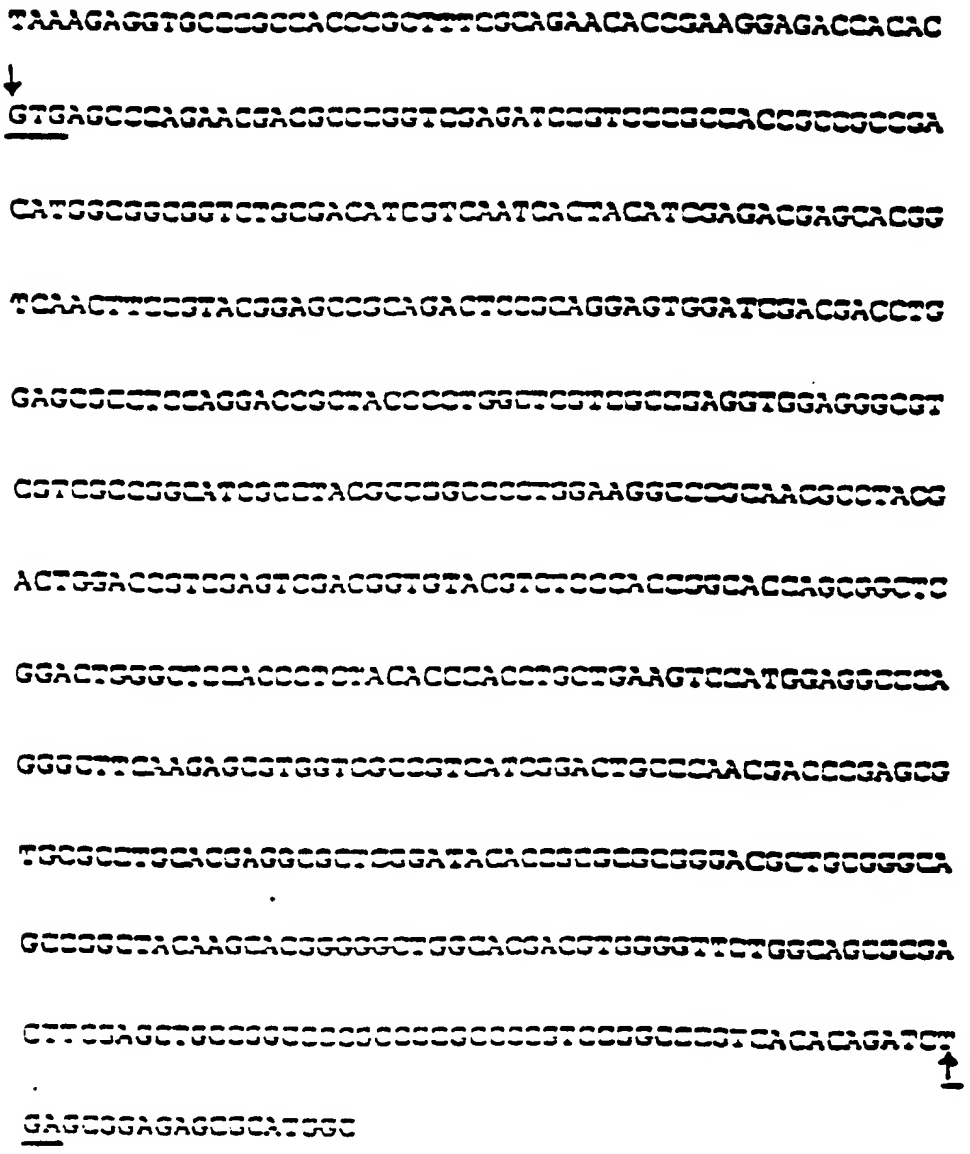
R G T L R A A G Y K H G G W H D V G

P W Q R D P E L P A P P R P V R P V

T Q I *

oder aus einem Teil der besagten Nucleotidsequenz mit ausreichender Länge, um für ein Polypeptid, das noch immer Phosphinotricin-acetyl-transferase-Aktivität hat, zu codieren.

2. Verfahren nach Anspruch 1, worin besagte DNS-Sequenz die folgende Nucleotidsequenz umfaßt:



oder einen Teil davon, der ein Polypeptid mit Phosphinotricin-acetyl-transferase-Aktivität, exprimiert.

3. Verfahren nach Anspruch 2, worin das Initiationscodon ATG eingesetzt wird für die Initiation CTG der besagten Nucleotidsequenz.

4. Verfahren zur Kontrolle der Vorgänge in Pflanzenzellen und Pflanzen, die solche Zellen eines Glutaminsynthetaseinhibitors umfassen, wenn die ersteren mit dem letzteren kontaktiert werden, das die stabile Integration in die genomische DNS der besagten Pflanzenzellen einer heterologen DNS umfaßt, einschließlich eines Promotors, der von Polymerasen der besagten Pflanzenzellen erkannt wird, und einer fremden Nucleotidsequenz die in der Form eines Proteins in den genannten pflanzenzellen exprimiert werden kann, und worin genanntes Protein eine enzymatische Aktivität hat, die die Inaktivierung oder Neutralisation des genannten Glutaminsynthetaseinhibitors verursachen kann, dadurch gekennzeichnet, daß die genannte fremde Nucleotidsequenz die Nucleotidsequenz, wie in einem der Ansprüche 1 bis 3 definiert, oder ein Teil davon ist.

5. Verfahren zur Herstellung einer Pflanze oder von Reproduktionsmaterial dieser Pflanze, die ein

heterologes genetisches Material einschließt, das stabil darin eingebaut ist und in besagten Pflanzen oder Reproduktionsmaterial in der Form eines Proteins exprimiert werden kann, das die Wirksamkeit eines Glutaminsynthetaseinhibitors inaktivieren oder neutralisieren kann, wobei dieses Verfahren das Transformieren von Zellen oder Gewebe der besagten Pflanzen mit einer DNS-Rekombinante umfaßt, die sowohl die heterologe DNS nach Anspruch 4, als auch die Regulationselemente enthält, ausgewählt aus jenen, die die stabile Integration besagter heterologer DNS in besagten Pflanzenzellen oder Gewebe verursachen und die Expression der besagten fremden Nucleotidsequenz in besagten Pflanzenzellen oder Pflanzengewebe ermöglichen können,
Regenerieren von Pflanzen oder Reproduktionsmaterial besagter Pflanzen oder von beiden aus Pflanzenzellen oder Gewebe, das mit besagter heterologer DNS transformiert wurde und, fakultativ, biologische Replikation besagter, zuletzt erwähnter Pflanzen oder von Reproduktionsmaterial oder von beiden, dadurch gekennzeichnet, daß besagte heterologe DNS die Nucleotidsequenz hat, die in einem der Ansprüche 1 bis 3 definiert ist, oder von besagtem Teil, der für ein Polypeptid mit Phosphinotricin-acetyl-transferase Aktivität codiert.

6. Verfahren nach Anspruch 5, worin die rekombinante DNS ein Vektor geeignet zur Transformation der Zellen besagter Pflanzen ist.

7. Verfahren nach Anspruch 5 oder 6, worin der Vektor eine Nucleotidsequenz umfaßt, die für ein Transitpeptid codiert eingeschoben zwischen besagtem Pflanzenpromotor-Bereich und besagtem rekombinanten DNS-Fragment.

8. Verfahren nach Anspruch 7, worin das Transitpeptid aus Ribulose-1,5-biphosphatcarboxylase und Chlorophyll-a/b-Bindungsproteinen ausgewählt ist.

9. Verfahren nach einem der Ansprüche 6 bis 8, worin besagter Vektor ein Ti-Plasmid ist.

10. Pflanzenzellen, nicht im wesentlichen biologisch transformiert, die stabil integriert in ihr Genom die heterologe Sequenz, wie in einem der Ansprüche 1 bis 3 definiert, besitzen.

11. Pflanzenzellen nach Anspruch 10, die zu einer Pflanze, die Samen produzieren kann, regeneriert werden können.

12. Pflanzenzellen nach einem der Ansprüche 10 oder 11, die nachweisbare Mengen Phosphinotricin-acetyl-transferase produzieren.

13. Samen, die stabil integriert in ihr Genom, eine DNS-Sequenz, wie in einem der Ansprüche 1 bis 3 definiert, besitzen.

14. Samen nach Anspruch 12, die zu einer Pflanze keimen können, die Samen produzieren kann, die eine nicht-sorten spezifische enzymatische Aktivität haben, die Glutaminsynthetaseinhibitoren inaktivieren oder neutralisieren kann.

15. Samen nach Anspruch 13 oder 14, die durch das Verfahren nach einem der Ansprüche 4 bis 8 transformiert werden.

16. Pflanzen, nicht im wesentlichen biologisch transformiert, die, stabil integriert in das Genom ihrer Zellen, eine heterologe DNS, wie in einem der Ansprüche 1 bis 3 definiert, besitzen.

17. Pflanzen nach Anspruch 16, die Samen produzieren können.

18. Pflanzen nach Anspruch 16 oder 17, die durch das Verfahren nach Anspruch 5 transformiert wurden.

19. Verfahren zum selektiven Schützen einer Pflanzenart und selektiven Vernichten von Unkraut auf einem Feld, das die Schritte des Behandelns eines Feldes mit einem Herbizid umfaßt, worin die Pflanzenarten in ihrem Genom eine heterologe DNS, wie in einem der Ansprüche 1 bis 3 definiert, enthalten und worin das verwendete Herbizid ein Glutaminsynthetaseinhibitor ist.

20. Verfahren nach Anspruch 18 oder 19, worin die Pflanzenarten gemäß dem Verfahren nach einem der Ansprüche 4 bis 8 transformiert werden.

21. Verfahren nach Anspruch 18 oder 19, worin eine Lösung eines Glutaminsynthetaseinhibitors auf das Feld nach dem Auswuchs der angebauten Pflanzenart mehrere Male aufgetragen wird, insbesondere nach Zeitabschnitten von etwa 20 bis 100 Tagen, bis früh- und spätkeimendes Unkraut vernichtet ist.

22. Verfahren nach Anspruch 18 oder 19, worin Glutaminsynthetaseinhibitoren Bialaphos, Phoshinotricin und verwandte Verbindungen umfassen.

23. Verfahren zum selektiven Schützen einer Pflanzenart auf einem Feld gegen Pilzkrankheiten, das die Schritte des Behandelns eines Feldes mit einem Herbizid umfaßt, das aus einem Glutaminsynthetaseinhibitor besteht, worin die Pflanzenarten in dem Genom ihrer Zellen eine heterologe DNS, wie in einem der Ansprüche 1 bis 3 definiert, enthalten und, worin das verwendete Herbizid ein Glutaminsynthetaseinhibitor ist.

24. Verfahren nach Anspruch 23, worin die Pflanzenarten gemäß dem Verfahren nach Anspruch 5 transformiert werden.

25. Verfahren nach Anspruch 23 oder 24, worin eine Lösung eines Glutaminsynthetaseinhibitors auf das Feld nach dem Auswuchs der angebauten Pflanzenarten mehrere Male aufgetragen wird, insbesondere nach Zeitabschnitten von etwa 20 bis 100 Tagen, bis die Pilze vernichtet sind.

26. Verfahren nach einem der Ansprüche 23 bis 25, worin der Glutaminsynthetaseinhibitor aus einer Gruppe, die Bialaphos, Phosphinotricin und verwandte Verbindungen umfaßt, ausgewählt ist.

27. Verfahren nach Anspruch 22 oder 26, worin Bialaphos, PPT oder die verwandte Verbindung bei einer Dosis im Bereich von etwa 0,4 bis etwa 1,6 kg/ha aufgetragen wird.

28. Verfahren nach Anspruch 27, worin Bialaphos, PPT oder die verwandte Verbindung in einem flüssigen Träger auf eine Konzentration verdünnt wird, die ihre Auftragung auf das Feld bei einer Menge im Bereich von etwa 2 l/ha bis etwa 8 l/ha ermöglicht.

29. Verfahren nach Anspruch 27 oder 28, worin selektiv geschützte Pflanzenarten Zuckerrübe, Reis, Kartoffel, Tomate, Mais und Tabak umfassen.

30. Verfahren zur Herstellung eines Vektors, der eine DNS-Sequenz, wie in einem der Ansprüche 1 bis 3 definiert, enthält und der für die Transformation von Pflanzenzellen und Pflanzen geeignet ist, das umfaßt, besagte DNS-Sequenz unter die Kontrolle eines Pflanzenpromotor-Bereichs und von Regulationselementen zu bringen, die besagter Vektor enthält, und, die die Expression besagter DNS-Sequenz in den Pflanzenzellen erlauben, wenn letztere später mit besagtem Vektor transformiert werden.

31. Verfahren nach Anspruch 30, worin besagte DNS-Sequenz den mit besagtem Vektor transformierten Pflanzenzellen Bialaphos-Resistenz verleiht.

32. Verfahren nach Anspruch 31, worin eine für ein Transitpeptid codierende Sequenz zwischen besagtem Pflanzenpromotor-Bereich und besagtem DNS-Fragment eingeschoben ist.

33. Verfahren nach Anspruch 32, worin das Transitpeptid aus Ribulose-1,5-biphosphatcarboxylase und Chlorophyll-a/b-Bindungsproteinen ausgewählt ist.

34. Verfahren nach einem der Ansprüche 30 bis 33, worin besagter Vektor ein modifiziertes Ti-Plasmid ist.

35. Verfahren nach Anspruch 30, worin besagte DNS-Sequenz unter der Kontrolle ist von, für die Transformation von Bakterien, insbesondere E. coli, geeigneten Replikationselementen.

Revendications

Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Fragment d'ADN pour la transformation ultérieure de cellules de plante, codant pour un polypeptide ayant une activité phosphinotricine acétyl transférase, qui consiste en une séquence de nucléotides codant pour la séquence suivante d'acides aminés :

V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N F

R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

H L L K S M E A Q G F K S V V A V I

G L P N D P S V R L H E A L G Y T A

R G T L R A A G Y K H G G W H D V G

F W Q R D F E L P A P P R P V R P V

T Q I *

ou une partie de cette séquence de nucléotides de longueur suffisante pour coder pour un polypeptide ayant encore une activité phosphinotricine acétyl transférase.

2. Fragment d'ADN suivant la revendication 1, qui comprend la séquence de nucléotides suivante :

TAAAGAGGTGCCCGCCACCCGCTTTCGCAGAACACCGAAGGAGACCACAC

GTGAGCCCAGAACGACGCCCGGTCGAGATCCGTCCCGCCACCGCCGCCGA

CATGGCGGCGGTCTGCGACATCGTCAATCACTACATCGAGACGAGCACGG

TCAACTTCCGTACGGAGCCGCAGACTCCGCAGGAGTGGATCGACGACCTG

GAGCGCCTCCAGGACCGCTACCCCTGGCTCGTCGCCGAGGTGGAGGGCGT

CGTCGCCGGCATCGCCTACGCCGGCCCCTGGAAGGCCCGCAACGCCTACG

ACTGGACCGTCGAGTCGACGGTGTACGTCTCCCACCGGCACCAGCGGCTC

GGACTGGGCTCCACCCTCTACACCCACCTGCTGAAGTCCATGGAGGCCCA

GGGCTTCAAGAGCGTGGTCGCCGTCATCGGACTGCCCAACGACCCGAGCG

TGCGCCTGCACGAGGCGCTCGGATACACCGCGCGCGGGACGCTGCGGGCA

GCCGGCTACAAGCACGGGGGCTGGCACGACGTGGGGTTCTGGCAGCGCGA

CTTCGAGCTGCCGGCCCCGCCCCGCCCCGTCCGGCCCGTCACACAGATCT

GAGCGGAGAGCGCATGGC

ou une partie de celle-ci exprimant un polypeptide ayant une activité phosphinotricine acétyl transférase.

3. Fragment d'ADN suivant la revendication 2, dans lequel le codon d'initiation ATG est substitué au codon d'initiation CTG.

4. Procédé pour contrôler l'action dans des cellules de plante et des plantes comprenant de telles cellules d'un inhibiteur de glutamine synthétase lorsque celles-ci sont mises en contact avec cet inhibiteur, qui comprend le fait de provoquer une intégration stable dans l'ADN génomique de ces cellules végétales d'un ADN hétérologue comprenant un promoteur reconnu par les polymérases de ces cellules de plante et une séquence nucléotidique étrangère capable d'être exprimée sous la forme d'une protéine dans ces cellules de plantes, et dans lequel cette protéine a une activité enzymatique capable de provoquer l'inactivation ou la neutralisation de cet inhibiteur de glutamine synthétase, caractérisé en ce que cette séquence nucléotidique étrangère est la séquence de nucléotides ou le fragment d'ADN suivant l'une quelconque des revendications 1 à 3.

5. Procédé pour produire une plante ou un matériel de reproduction de cette plante comprenant un matériel génétique hétérologue intégré de façon stable dans ceux-ci et capable d'être exprimé dans ces plantes ou ce matériel de reproduction sous la forme d'une protéine capable d'inactiver ou de

neutraliser l'activité d'un inhibiteur de glutamine synthétase, lequel procédé comprend la transformation de cellules ou de tissu de ces plantes avec un ADN recombinant contenant un ADN hétérologue, ainsi que les éléments régulateurs sélectionnés parmi ceux qui sont capables de provoquer l'intégration stable de cet ADN hétérologue dans ces cellules ou tissu de plante et de permettre l'expression de cette séquence nucléotidique étrangère dans ces cellules de plante ou tissu de plante, la régénération des plantes ou du matériel de reproduction de ces plantes ou des deux à partir des cellules ou du tissu de plante transformés avec cet ADN hétérologue et, éventuellement, la réplication biologique de ces plantes ou du matériel de reproduction mentionnés en dernier ou des deux, caractérisé en ce que cet ADN hétérologue a la séquence nucléotidique du fragment d'ADN suivant l'une quelconque des revendications 1 à 3 ou de cette partie exprimant un polypeptide ayant une activité phosphinotricine acétyl transférase.

6. Procédé suivant la revendication 5, dans lequel l'ADN recombinant est un vecteur convenable pour la transformation des cellules de cette plante.

7. Procédé suivant les revendications 5 ou 6, dans lequel le vecteur comprend une séquence nucléotidique codant pour un peptide transitoire intercalée entre cette région promoteur de la plante et ce fragment d'ADN recombinant.

8. Procédé suivant la revendication 7, dans lequel le peptide transitoire est choisi parmi la ribulose-1,5-biphosphate carboxylase et les protéines de liaison des chlorophylles a/b.

9. Procédé suivant l'une quelconque des revendications 6 à 8, dans lequel ce vecteur est un plasmide Ti.

10. Cellules de plante, transformées de façon essentiellement non biologique, qui possèdent l'ADN hétérologue suivant l'une quelconque des revendications 1 à 3 intégré de façon stable dans leur génome.

11. Cellules de plante suivant la revendication 10, qui peuvent être régénérées en une plante capable de produire des semences.

12. Cellules de plante suivant l'une quelconque des revendications 10 et 11, qui produisent des quantités détectables de phosphinotricine acétyl transférase.

13. Semences, qui possèdent un fragment d'ADN suivant l'une quelconque des revendications 1 à 3 intégré de façon stable dans leur génome.

14. Semences suivant la revendication 12, qui sont capables de germer en une plante capable de produire des semences ayant une activité enzymatique non spécifique d'une variété capable d'inactiver ou de neutraliser des inhibiteurs de glutamine synthétase.

15. Semences suivant les revendications 13 ou 14, qui sont transformées par le procédé suivant l'une quelconque des revendications 4 à 8.

16. Plantes, transformées de façon essentiellement non biologique, qui possèdent un ADN hétérologue suivant l'une quelconque des revendications 1 à 3 intégré de façon stable dans le génome de leurs cellules.

17. Plantes suivant la revendication 16, qui sont capables de produire des semences.

18. Plantes suivant les revendications 16 ou 17, qui sont transformées suivant le procédé de la revendication 5.

19. Procédé pour protéger sélectivement une espèce végétale et détruire sélectivement des mauvaises herbes dans un champ, qui comprend les étapes de traitement du champ avec un herbicide, dans lequel l'espèce végétale contient dans son génome un ADN hétérologue suivant l'une quelconque des revendications 1 à 3, et dans lequel l'herbicide utilisé est un inhibiteur de glutamine synthétase.

20. Procédé suivant les revendications 18 ou 19, dans lequel les espèces végétales sont transformées suivant le procédé de l'une quelconque des revendications 4 à 8.

21. Procédé suivant les revendications 18 ou 19, dans lequel une solution d'un inhibiteur de glutamine synthétase est appliquée sur le champ après émergence de l'espèce végétale cultivée, à plusieurs reprises, en particulier à des intervalles de temps d'environ 20 et 100 jours, jusqu'à ce que les mauvaises herbes à germination précoce ou tardive soient détruites.

22. Procédé suivant les revendications 18 ou 19, dans lequel les inhibiteurs de glutamine synthétase comprennent le Bialaphos, la phosphinotricine et des composés apparentés.

23. Procédé pour protéger sélectivement une espèce végétale dans un champ contre des maladies fongiques comprenant les étapes de traitement du champ avec un herbicide consistant en un inhibiteur de glutamine synthétase, dans lequel l'espèce végétale contient dans le génome de ses cellules un ADN hétérologue suivant l'une des revendications 1 à 3 et dans lequel l'herbicide utilisé est un inhibiteur de glutamine synthétase.

24. Procédé suivant la revendication 23, dans lequel les espèces végétales sont transformées suivant le procédé de la revendication 5.

25. Procédé suivant les revendications 23 ou 24, dans lequel une solution d'un inhibiteur de glutamine synthétase est appliquée sur le champ, après émergence de l'espèce végétale cultivée, à plusieurs reprises, en particulier à des intervalles de temps d'environ 20 et 100 jours, jusqu'à ce que les champignons soient détruits.

26. Procédé suivant l'une quelconque des revendications 23 à 25, dans lequel l'inhibiteur de glutamine synthétase est choisi dans le groupe consistant en Bialaphos, phosphinotricine et des composés apparentés.

27. Procédé suivant les revendications 22 ou 26, dans lequel le Bialaphos, la phosphinotricine ou le composé apparenté est appliqué à une dose comprise entre environ 0,4 et 1,6 kg/ha.

28. Procédé suivant la revendication 27, dans lequel le Bialaphos, la phosphinotricine ou le composé apparenté sont dilués dans un support liquide à une concentration permettant leur application dans le champ à un taux compris entre environ 2 l/ha et environ 8 l/ha.

29. Procédé suivant les revendications 27 ou 28, dans lequel les espèces végétales protégées sélectivement comprennent la betterave à sucre, le riz, la pomme de terre, la tomate, le maïs et le tabac.

30. Vecteur, qui comprend un fragment d'ADN suivant l'une quelconque des revendications 1 à 3.

31. Vecteur suivant la revendication 30, qui convient pour la transformation de cellules de plante et de plantes.

32. Vecteur suivant la revendication 31, dans lequel ce fragment d'ADN est sous le contrôle d'une région promoteur de la plante et d'éléments de régulation permettant l'expression de ces fragments d'ADN dans les cellules de plante, lorsque ces dernières sont ensuite transformées avec ce vecteur.

33. Vecteur suivant la revendication 32, qui confère une résistance au Bialaphos aux cellules de plante transformées.

34. Vecteur suivant la revendication 33, dans lequel une séquence codant pour un peptide transitoire est intercalée entre cette région promoteur de la plante et ce fragment d'ADN.

35. Vecteur suivant la revendication 34, dans lequel ce peptide transitoire est choisi parmi la ribulose-1,5-biphosphate carboxylase et les protéines de liaison des chlorophylles a/b.

36. Vecteur suivant l'une quelconque des revendications 31 à 35, dans lequel ce vecteur est un plasmide

Ti modifié.

37. Vecteur suivant la revendication 29, dans lequel ce fragment d'ADN est sous le contrôle des éléments d'un réplicon convenable pour la transformation des bactéries, en particulier E. coli.

Revendications pour les Etats contractants suivants : AT, ES

1. Procédé pour la transformation de cellules de plante, qui comprend la transformation de ces cellules de plante avec un vecteur convenable pour la transformation des cellules de plante et qui contient une séquence d'ADN codant pour un polypeptide ayant une activité phosphinotricine acétyl transférase, qui consiste en une séquence de nucléotides codant pour la séquence suivante d'acides aminés :

V S P E R R P V E I R P A T A A D M

A A V C D I V N H Y I E T S T V N F

R T E P Q T P Q E W I D D L E R L Q

D R Y P W L V A E V E G V V A G I A

Y A G P W K A R N A Y D W T V E S T

V Y V S H R H Q R L G L G S T L Y T

H L L K S M E A Q G F K S V V A V I

G L P N D P S V R L H E A L G Y T A

R G T L R A A G Y K H G G W H D V G

F W Q R D F E L P A P P R P V R P V

T Q I *

ou une partie de cette séquence de nucléotides de longueur suffisante pour coder pour un polypeptide ayant encore une activité phosphinotricine acétyl transférase.

2. Procédé suivant la revendication 1, dans lequel cette séquence d'ADN comprend la séquence de nucléotides suivante :

TAAAGAGGTGCCCGCCACCCGCTTTCGCAGAACACCGAAGGAGACCACAC

GTGAGCCCAGAACGACGCCCGGTCGAGATCCGTCCCGCCACCGCCGCCGA

CATGGCGGCGGTCTGCGACATCGTCAATCACTACATCGAGACGAGCACGG

TCAACTTCCGTACGGAGCCGCAGACTCCGCAGGAGTGGATCGACGACCTG

GAGCGCCTCCAGGACCGCTACCCCTGGCTCGTCGCCGAGGTGGAGGGCGT

CGTCGCCGGCATCGCCTACGCCGGCCCCTGGAAGGCCCGCAACGCCTACG

ACTGGACCGTCGAGTCGACGGTGTACGTCTCCCACCGGCACCAGCGGCTC

GGACTGGGCTCCACCCTCTACACCCACCTGCTGAAGTCCATGGAGGCCCA

GGGCTTCAAGAGCGTGGTCGCCGTCATCGGACTGCCCAACGACCCGAGCG

TGCGCCTGCACGAGGCGCTCGGATACACCGCGCGCGGGACGCTGCGGGCA

GCCGGCTACAAGCACGGGGGCTGGCACGACGTGGGGTTCTGGCAGCGCGA

CTTCGAGCTGCCGGCCCCGCCCCGCCCCGTCCGGCCCGTCACACAGATCT

GAGCGGAGAGCGCATGGC

ou une partie de celle-ci exprimant un polypeptide ayant une activité phosphinotricine acétyl transférase.

3. Procédé suivant la revendication 2, dans lequel le codon d'initiation ATG est substitué au codon d'initiation CTG.

4. Procédé pour contrôler l'action dans des cellules de plante et des plantes comprenant de telles cellules d'un inhibiteur de glutamine synthétase lorsque celles-ci sont mises en contact avec cet inhibiteur, qui comprend le fait de provoquer une intégration stable dans l'ADN génomique de ces cellules végétales d'un ADN hétérologue comprenant un promoteur reconnu par les polymérases de ces cellules de plante et une séquence nucléotidique étrangère capable d'être exprimée sous la forme d'une protéine dans ces cellules de plantes, et dans lequel cette protéine a une activité enzymatique capable de provoquer l'inactivation ou la neutralisation de cet inhibiteur de glutamine synthétase, caractérisé en ce que cette séquence nucléotidique étrangère est la séquence de nucléotides définies dans l'une quelconque des revendications 1 à 3 ou une partie de celle-ci.

5. Procédé pour produire une plante ou un matériel de reproduction de cette plante comprenant un matériel génétique hétérologue intégré de façon stable dans ceux-ci et capable d'être exprimé dans ces plantes ou ce matériel de reproduction sous la forme d'une protéine capable d'inactiver ou de neutraliser l'activité d'un inhibiteur de glutamine synthétase, lequel procédé comprend la transformation de cellules ou de tissu de ces plantes avec un ADN recombinant contenant l'ADN hétérologue de la revendication 4, ainsi que les éléments régulateurs sélectionnés parmi ceux qui sont capables de provoquer l'intégration stable de cet ADN hétérologue dans ces cellules ou tissu de plante et de permettre l'expression de cette séquence nucléotidique étrangère dans ces cellules de plante ou tissu de plante, la régénération des plantes ou du matériel de reproduction de ces plantes ou des deux à partir des cellules ou du tissu de plante transformés avec cet ADN hétérologue et, éventuellement, la réplication biologique de ces plantes ou du matériel de reproduction mentionnés en dernier ou des deux, caractérisé en ce que cet ADN hétérologue a la séquence nucléotidique définie dans l'une quelconque des revendications 1 à 3 ou cette partie qui code pour un polypeptide ayant une activité phosphinotricine acétyl transférase.

6. Procédé suivant la revendication 5, dans lequel l'ADN recombinant est un vecteur convenable pour la transformation des cellules de cette plante.

7. Procédé suivant les revendications 5 ou 6, dans lequel le vecteur comprend une séquence nucléotidique codant pour un peptide transitoire intercalée entre cette région promoteur de la plante et ce fragment d'ADN recombinant.

8. Procédé suivant la revendication 7, dans lequel le peptide transitoire est choisi parmi la ribulose-1,5-biphosphate carboxylase et les protéines de liaison des chlorophylles a/b.

9. Procédé suivant l'une quelconque des revendications 6 à 8, dans lequel ce vecteur est un plasmide Ti.

10. Cellules de plante, transformées de façon essentiellement non biologique, qui possèdent la séquence hétérologue définie dans l'une quelconque des revendications 1 à 3 intégrée de façon stable dans leur génome.

11. Cellules de plante suivant la revendication 10, qui peuvent être régénérées en une plante capable de produire des semences.

12. Cellules de plante suivant l'une quelconque des revendications 10 et 11, qui produisent des quantités détectables de phosphinotricine acétyl transférase.

13. Semences, qui possèdent une séquence d'ADN suivant l'une quelconque des revendications 1 à 3 intégrée de façon stable dans leur génome.

14. Semences suivant la revendication 12, qui sont capables de germer en une plante capable de produire des semences ayant une activité enzymatique non spécifique d'une variété capable d'inactiver ou de neutraliser des inhibiteurs de glutamine synthétase.

15. Semences suivant les revendications 13 ou 14, qui sont transformées par le procédé suivant l'une quelconque des revendications 4 à 8.

16. Plantes, transformées de façon essentiellement non biologique, qui possèdent un ADN hétérologue suivant l'une quelconque des revendications 1 à 3 intégré de façon stable dans le génome de leurs cellules.

17. Plantes suivant la revendication 16, qui sont capables de produire des semences.

18. Plantes suivant les revendications 16 ou 17, qui sont transformées suivant le procédé de la revendication 5.

19. Procédé pour protéger sélectivement une espèce végétale et détruire sélectivement des mauvaises herbes dans un champ, qui comprend les étapes de traitement du champ avec un herbicide, dans

lequel l'espèce végétale contient dans son génome un ADN hétérologue suivant l'une quelconque des revendications 1 à 3, et dans lequel l'herbicide utilisé est un inhibiteur de glutamine synthétase.

20. Procédé suivant les revendications 18 ou 19, dans lequel les espèces végétales sont transformées suivant le procédé de l'une quelconque des revendications 4 à 8.

21. Procédé suivant les revendications 18 ou 19, dans lequel une solution d'un inhibiteur de glutamine synthétase est appliquée sur le champ, après émergence de l'espèce végétale cultivée, à plusieurs reprises, en particulier à des intervalles de temps d'environ 20 et 100 jours, jusqu'à ce que les mauvaises herbes à germination précoce ou tardive soient détruites.

22. Procédé suivant les revendications 18 ou 19, dans lequel les inhibiteurs de glutamine synthétase comprennent le Bialaphos, la phosphinotricine et des composés apparentés.

23. Procédé pour protéger sélectivement une espèce végétale dans un champ contre des maladies fongiques comprenant les étapes de traitement du champ avec un herbicide consistant en un inhibiteur de glutamine synthétase, dans lequel l'espèce végétale contient dans le génome de ses cellules un ADN hétérologue suivant l'une des revendications 1 à 3 et dans lequel l'herbicide utilisé est un inhibiteur de glutamine synthétase.

24. Procédé suivant la revendication 23, dans lequel les espèces végétales sont transformées suivant le procédé de la revendication 5.

25. Procédé suivant les revendications 23 ou 24, dans lequel une solution d'un inhibiteur de glutamine synthétase est appliquée sur le champ, après émergence de l'espèce végétale cultivée, à plusieurs reprises, en particulier à des intervalles de temps d'environ 20 et 100 jours, jusqu'à ce que les champignons soient détruits.

26. Procédé suivant l'une quelconque des revendications 23 à 25, dans lequel l'inhibiteur de glutamine synthétase est choisi dans le groupe consistant en Bialaphos, phosphinotricine et des composés apparentés.

27. Procédé suivant les revendications 22 ou 26, dans lequel le Bialaphos, la phosphinotricine ou le composé apparenté est appliqué à une dose comprise entre environ 0,4 et 1,6 kg/ha.

28. Procédé suivant la revendication 27, dans lequel le Bialaphos, la phosphinotricine ou le composé apparenté sont dilués dans un support liquide à une concentration permettant leur application dans le champ à un taux compris entre environ 2 l/ha et environ 8 l/ha.

29. Procédé suivant les revendications 27 ou 28, dans lequel les espèces végétales protégées sélectivement comprennent la betterave à sucre, le riz, la pomme de terre, la tomate, le maïs et le tabac.

30. Procédé pour produire un vecteur, qui contient une séquence d'ADN suivant l'une quelconque des revendications 1 à 3 et qui convient pour la transformation de cellules de plante et de plantes, qui comprend la mise de cette séquence d'ADN sous le contrôle d'une région promoteur d'une plante, et d'éléments de régulation contenus dans ce vecteur et qui permettent l'expression de cette séquence d'ADN dans les cellules de plante, lorsque ces dernières sont ensuite transformées avec ce vecteur.

31. Procédé suivant la revendication 30, dans lequel cette séquence d'ADN confère une résistance au Bialaphos aux cellules de plante transformées avec ce vecteur.

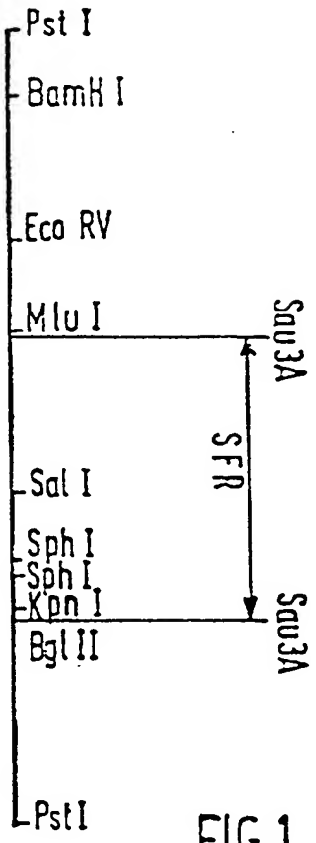
32. Procédé suivant la revendication 31, dans lequel une séquence codant pour un peptide transitoire est intercalée entre cette région promoteur de la plante et ce fragment d'ADN.

33. Procédé suivant la revendication 32, dans lequel ce peptide transitoire est choisi parmi la ribulose-1,5-biphosphate carboxylase et les protéines de liaison des chlorophylles a/b.

34. Vecteur suivant l'une quelconque des revendications 30 à 33, dans lequel ce vecteur est un plasmide

Ti modifié.

35. Procédé suivant la revendication 30, dans lequel cette séquence d'ADN est sous le contrôle des éléments d'un réplicon convenable pour la transformation des bactéries, en particulier E. coli.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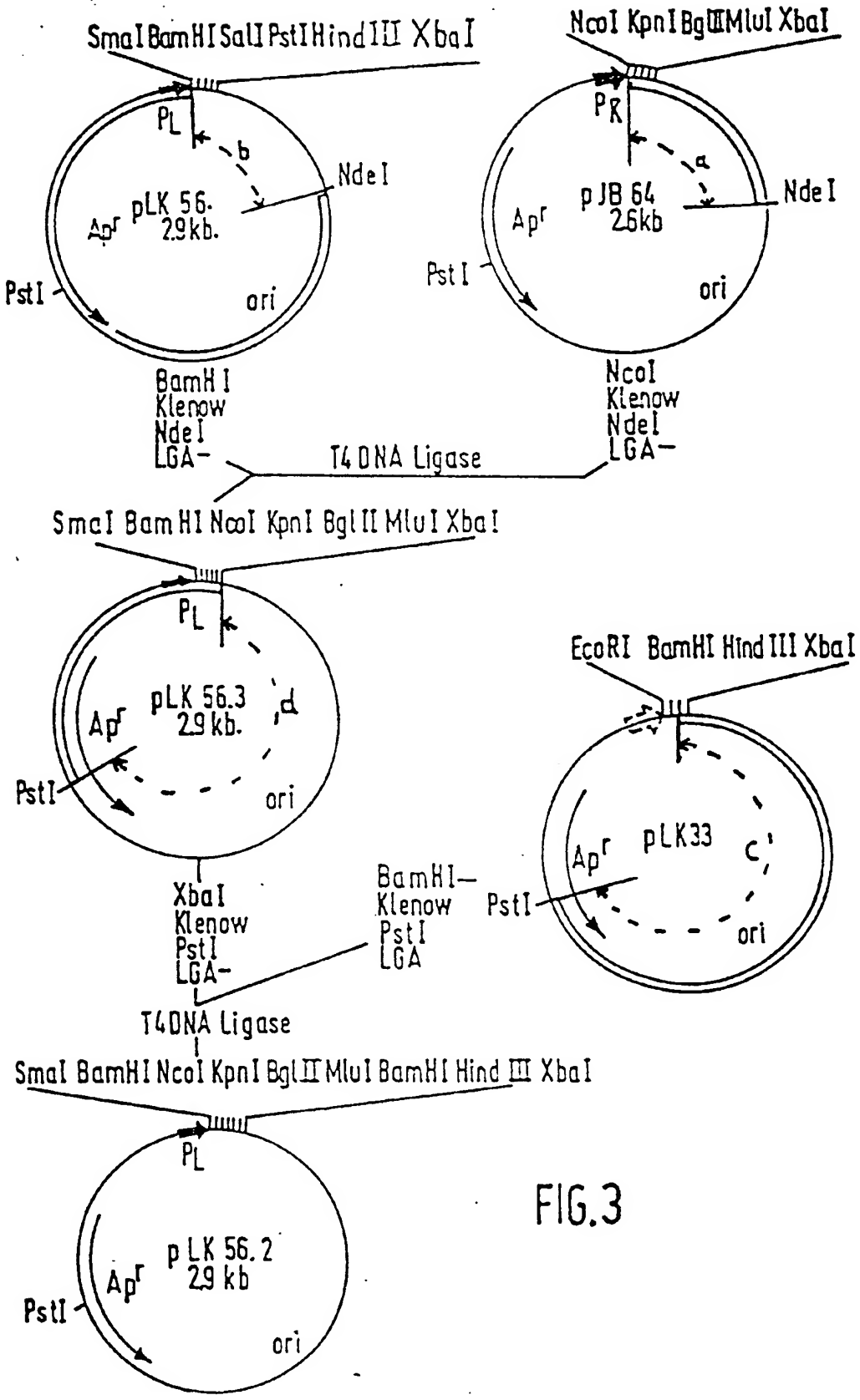
BamH I
Eco RV
Mlu I
Sau3A
SFR
Sal I
Sph I
Sph I
Kpn I
Sau3A
Bgl II
PstI

FIG.1

CCC GCT CAA GCT CGC TGT CAT TTT CGA GAC GCC ATC TTT GGA AGC
GGT GGC CGA ATC CGT ACT GCG CGG ACT CGA CGA CGC GTA AAA CGA
TCG ACC ACG TAC ACG AGT CCG GAC ACG GGG CGA GGA GGC CCG GTT
CCG GCA CCG AGG AAG ACC GAA GGA AGA CCA CAC GTG AGC CCA GAA
CGA CGC CCG GCC GAC ATC CGC CGT GCC ACC GAG GCG GAC ATG CCG
FokI
GCG GTC TGC ACC ATC GTC AAC CAC TAC ATC GAG ACA AGC ACG GTC
AAC TTC CGT ACC GAG CCG CAG GAA CCG CAG GAG TGG ACG GAC GAC
CTC GTC CGT CTG CGG GAG CGC TAT CCC TGG CTC GTC GCC GAG GTG
GAC GGC GAG GTC GCC GGC ATC GCC TAC GCG GGC CCC TGG AAG GCA
CGC AAC GCC TAC GAC TGG ACG GCC GAG TCG ACC GTG TAC GTC TCC
CCC CGC CAC CAG CGG ACG GGA CTG GGC TCC ACG CTC TAC ACC CAC
CTG CTG AAG TCC CTG GAG GCA CAG GGC TTC AAG AGC GTG GTC GCT
GTC ATC GGG CTG CCC AAC GAC CCG AGC GTG CGC ATG CAC GAG GCG
CTC GGA TAT GCC CCC CGC GGC ATG CTG CGG GCG GCC GGC TTC AAG
CAC GGG AAC TGG CAT GAC GTG GGT TTC TGG CAG CTG GAC TTC AGC
CTG CCG GTA CCG CCC CGT CCG GTC CTG CCC GTC ACC GAG ATC TGA
BglII
ACG GAG TGC GCG TGG GCA TCG CCC GAG TTG GAG CTG GTA CGG GAA
CTC ATC GAA CTC AAC TGG CAT ACC CGC AAT GGT GAG GTG GAA CCG
CGG CGG ATC GCG TAC GAC CGT GCC CAG G

FIG.2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PL
b
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pLK 56.
2.9kb.
Apr
PstI
ori
BamH I
Klenow
NdeI
LGA-
NcoI KpnI BglII MluI XbaI
PR
a
pJB 64
2.6kb
Apr
NdeI
PstI
ori
NcoI
Klenow
NdeI
LGA-
T4 DNA Ligase
SmaI BamHI NcoI KpnI BglII MluI XbaI
PL
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2.9 kb.
d
Apr
PstI
ori
XbaI
Klenow
PstI
LGA-
EcoRI BamHI HindIII XbaI
pLK33
c
Apr
PstI
ori
BamHI-
Klenow
PstI
LGA
T4DNA Ligase
SmaI BamHI NcoI KpnI BglII MluI BamHI HindIII XbaI
PL
pLK 56.2
2.9 kb
Apr
PstI
ori

FIG.3

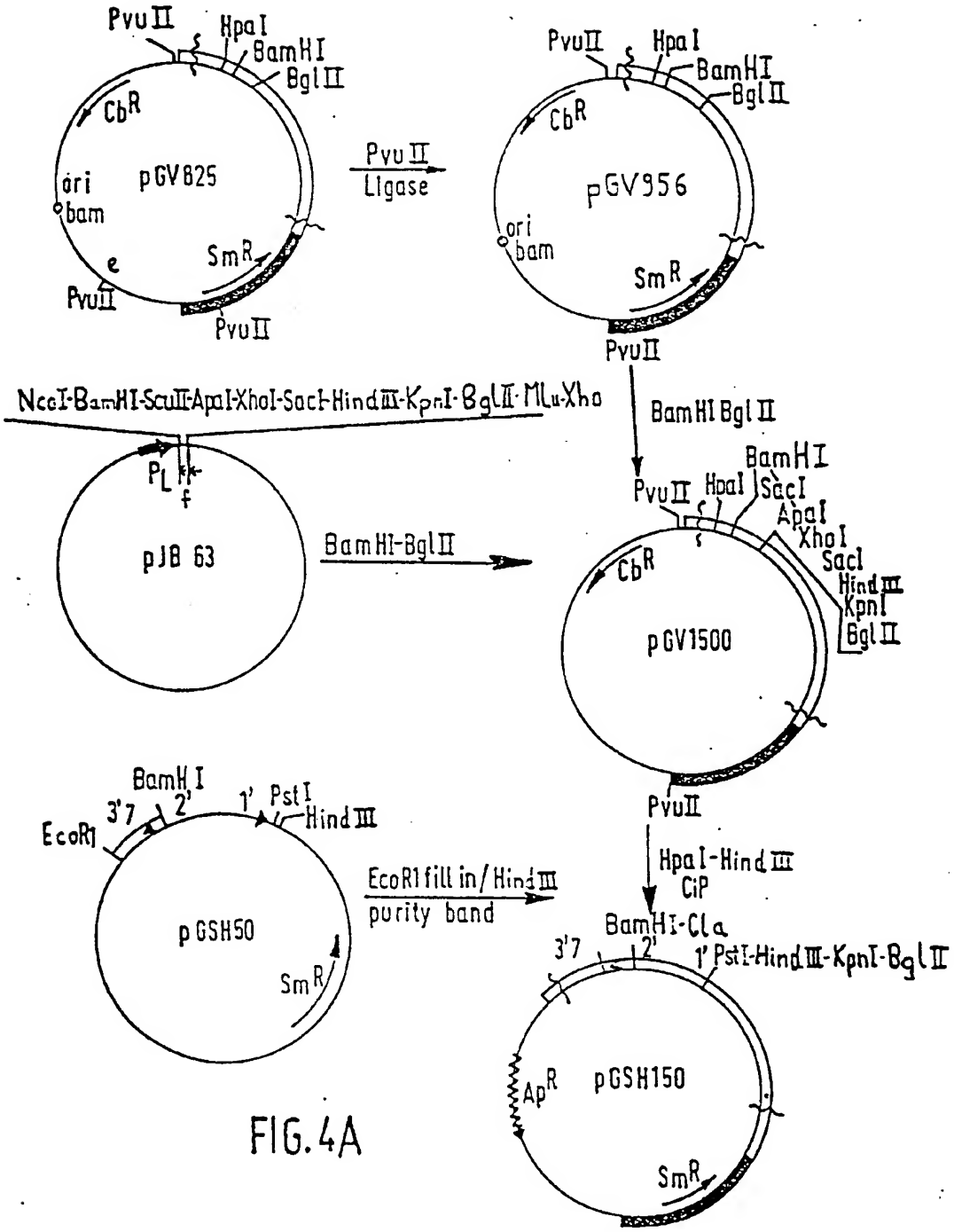

PvuII
HpaI
BamHI
BglII
CbR
pGV825
ori
bam
SmR
PvuII
PvuII Ligase
pGV956
NcoI-BamHI-ScuII-ApaI-XhoI-SacI-HindIII-KpnI-BglII-MluI-Xho
PL
f
pJB 63
BamHI-BglII
BamHI BglII
SacI
ApaI
XhoI
HindIII
KpnI
pGV1500
BamHI
3'7
2'
1'
PstI
HindIII
EcoRI
pGSH50
EcoRI fill in/HindIII purity band
HpaI-HindIII CiP
BamHI-ClaI
1'PstI-HindIII-KpnI-BglII
ApR
pGSH150

FIG.4A

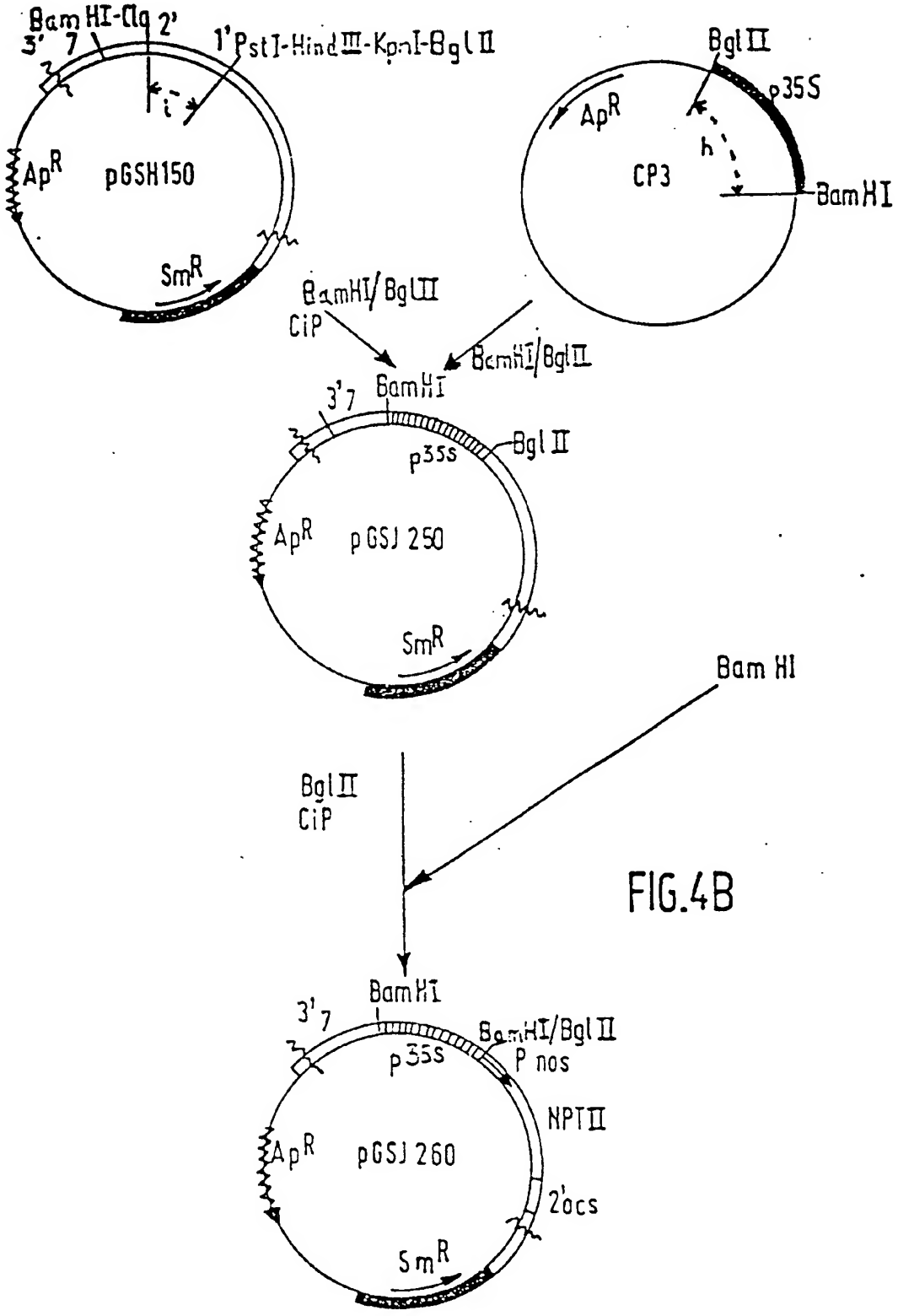
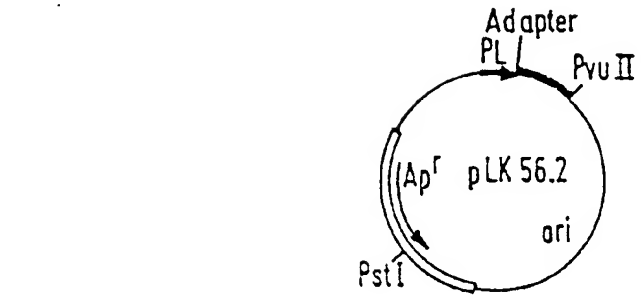

BamHI-Cla
3'
7
2'
1'
PstI-HindIII-KpnI-BglII
i
ApR
pGSH150
SmR
BglII
p35S
ApR
h
CP3
BamHI
BamHI/BglII
CiP
BamHI/BglII
3'
7
BamHI
p35s
BglII
ApR
pGSJ 250
SmR
Bam HI
BglII
CiP
BamHI
3'
7
BamHI/BglII
p35s
P nos
NPTII
ApR
pGSJ 260
2'ocs
SmR

FIG.4B



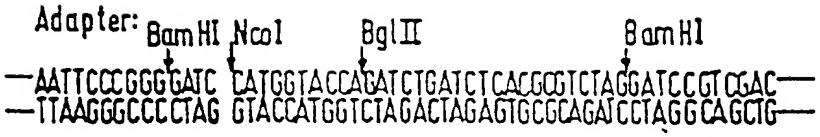


FIG.5A

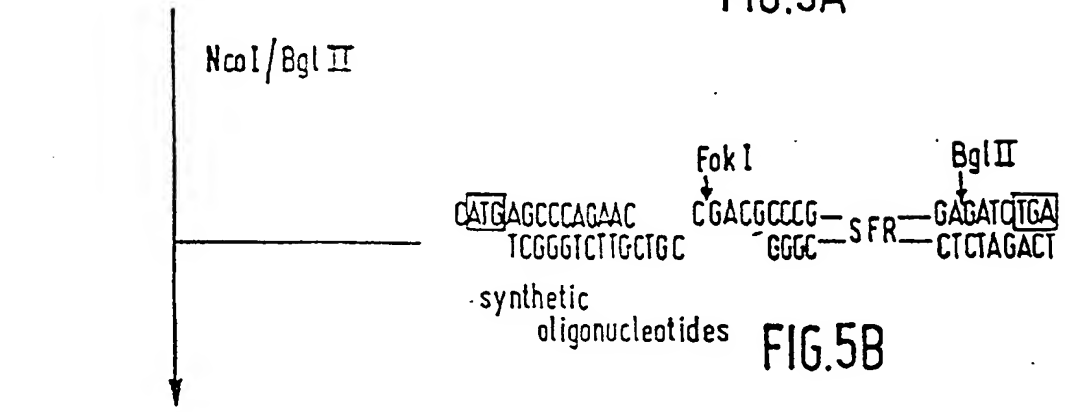


FIG.5B

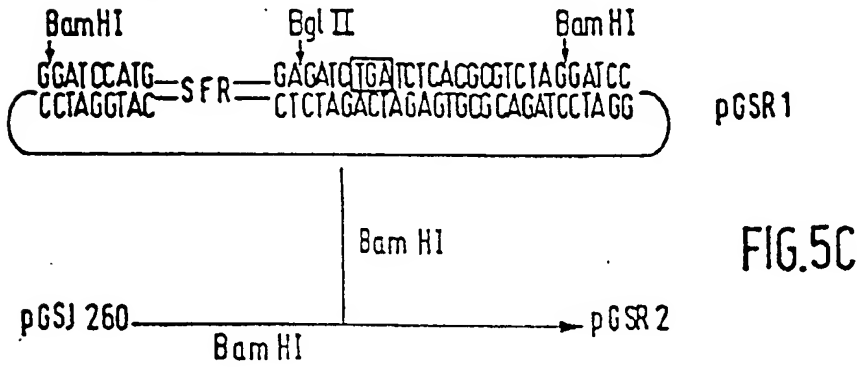


FIG.5C

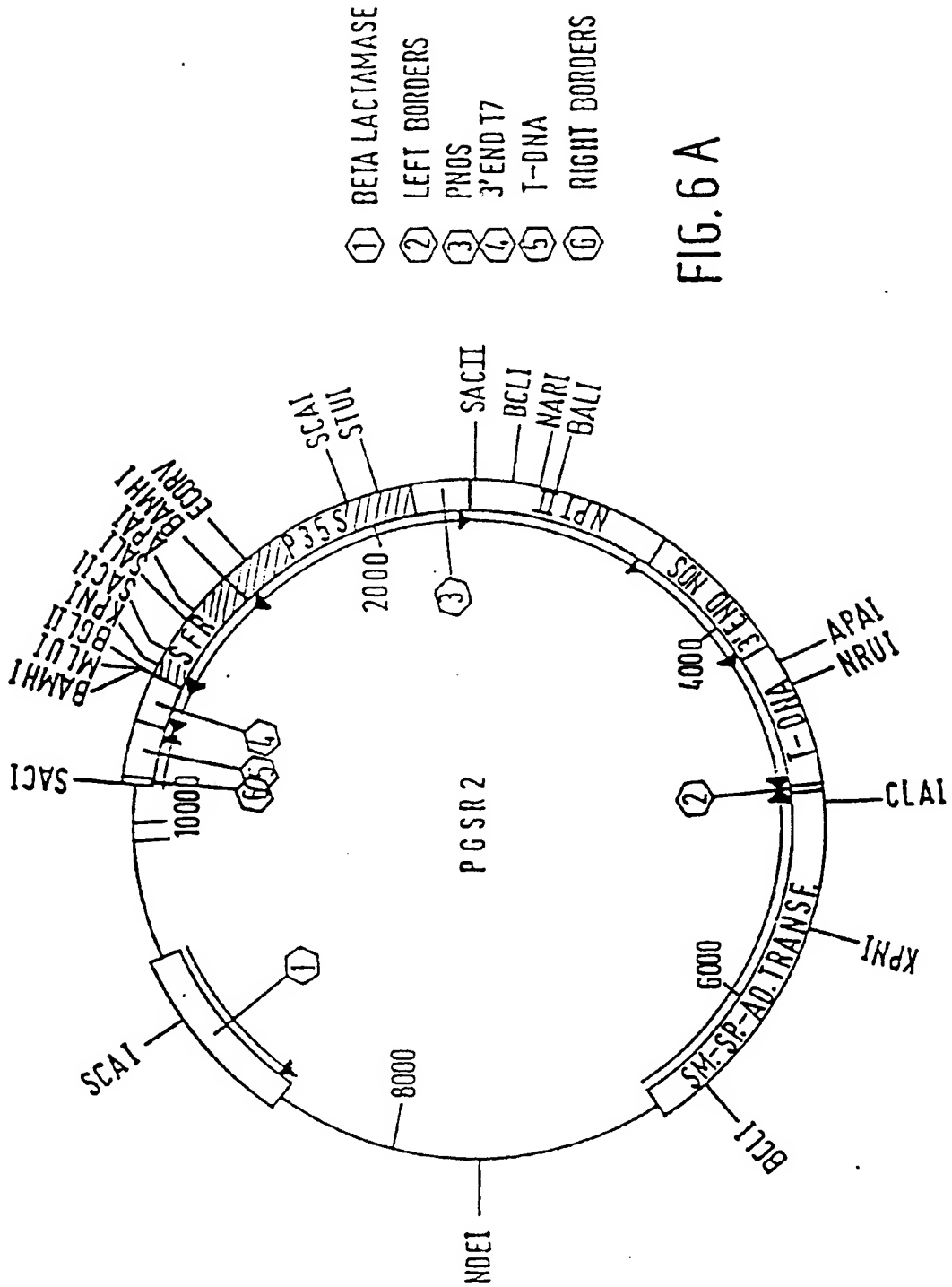

① BETA LACTAMASE
② LEFT BORDERS
③ PNOS
④ 3'END T7
⑤ T-DNA
⑥ RIGHT BORDERS
SCA1
STU1
SACII
BCLI
NARI
BALI
NPT II
3'END NOS
T-DNA
APAI
NRUI
CLAI
KPNI
BCLI
SM-SP-AD.TRANSF.
6000
NDEI
8000
SCAI
SACI
1000
P G S R 2
2000
P35S
EcoRV
BAMHI
SALI
SACII
XPNI
BGLII
MLUI
BAMHI
4000

FIG. 6 A

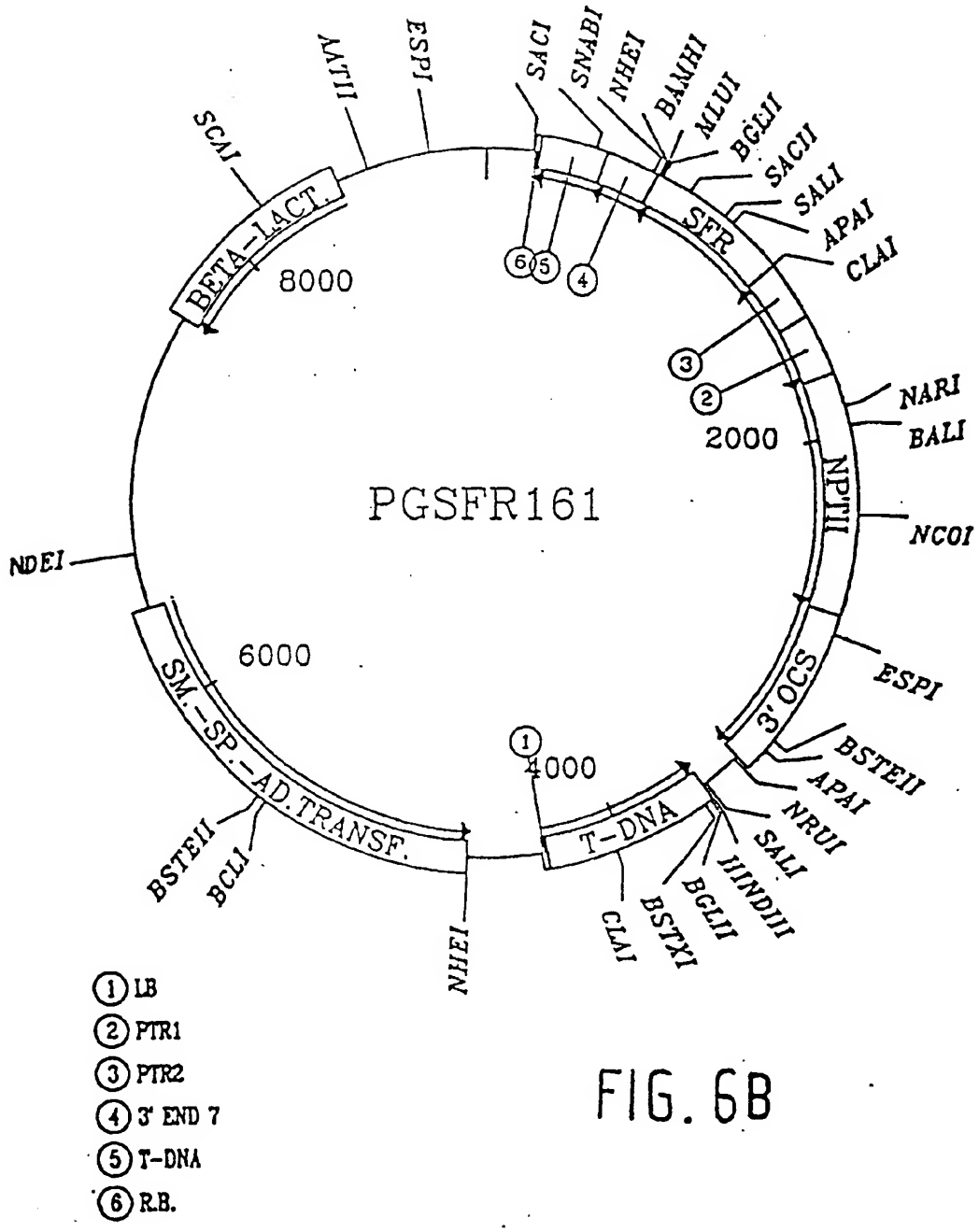

PGSFR161
2000
4000
6000
8000
BETA-LACT.
SM.-SP.-AD.TRANSF.
T-DNA
SFR
NPTIII
3'OCS
SCAI
AVII
ESPI
SACI
SNABI
NHEI
BAMHI
MLUI
BGLII
SACII
SALI
APAI
CLAI
NARI
BALI
NCOI
ESPI
BSTEII
APAI
NRUI
SALI
HINDIII
BGLII
BSTXI
CLAI
NHEI
BCLI
BSTEII
NDEI
1 LB
2 PTR1
3 PTR2
4 3' END 7
5 T-DNA
6 R.B.

FIG. 6B

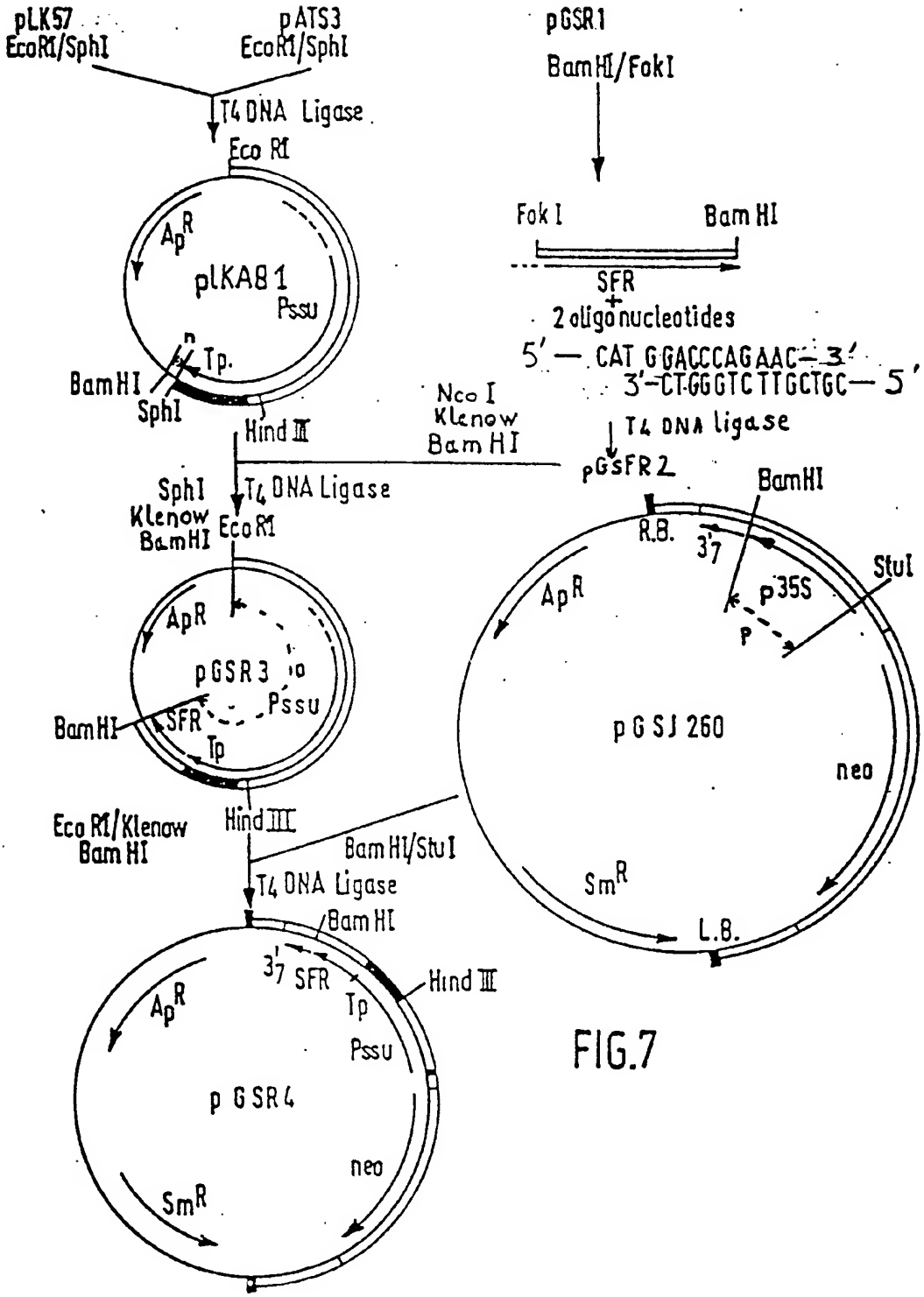

pLK57
EcoRI/SphI
pATS3
EcoRI/SphI
T4 DNA Ligase
Eco RI
ApR
pLKA81
Pssu
Tp
BamHI
SphI
Hind III
pGSR1
BamHI/FokI
Fok I
Bam HI
SFR
+
2 oligonucleotides
5' — CAT G GACCCAGAAC — 3'
3'-CT GGGTCTTGCTGC — 5'
Nco I
Klenow
Bam HI
T4 DNA ligase
pGSFR2
SphI
Klenow
BamHI
T4 DNA Ligase
EcoRI
ApR
pGSR3
Pssu
SFR
Tp
BamHI
R.B.
3'7
BamHI
ApR
p35S
StuI
pGSJ260
neo
SmR
L.B.
EcoRI/Klenow
BamHI
Hind III
BamHI/StuI
T4 DNA Ligase
BamHI
3'7 SFR
Hind III
ApR
Tp
Pssu
pGSR4
neo
SmR

FIG.7

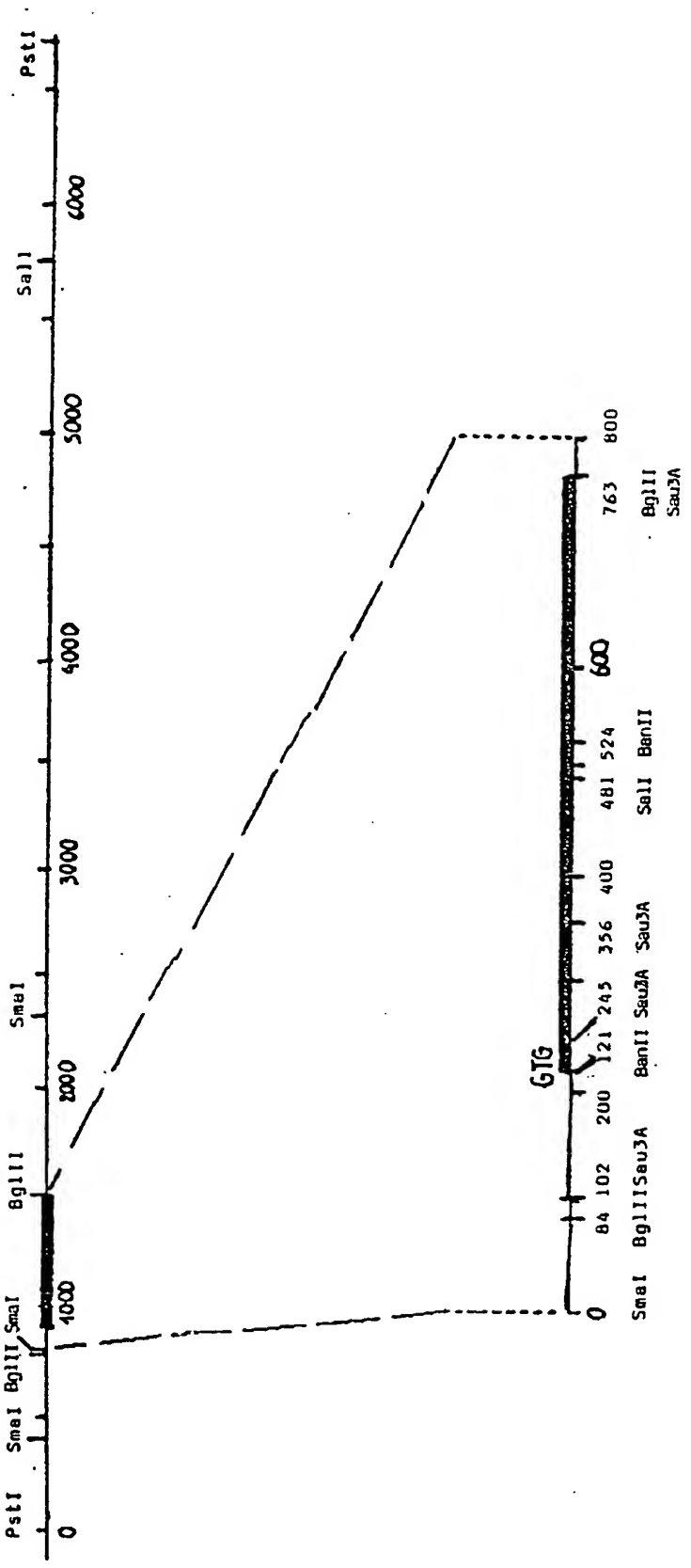

PstI
SmaI BglII
SmaI
BglII
SmaI
SalI
PstI
0
1000
2000
3000
4000
5000
6000
GTG
0
84 102
200
121 245
356
400
481 524
600
763
800
SmaI BglIISau3A
BanII Sau3A
Sau3A
SalI BanII
BglII
Sau3A

FIGURE 8

TAAAGAGGTGCCCGCCACCCGCTTTCGCAGAACACCGAAGGAGACCACAC

GTGAGCCCAGAACGACGCCCGGTCGAGATCCGTCCCGCCACCGCCGCCGA

CATGGCGGCGGTCTGCGACATCGTCAATCACTACATCGAGACGAGCACGG

TCAACTTCCGTACGGAGCCGCAGACTCCGCAGGAGTGGATCGACGACCTG

GAGCGCCTCCAGGACCGCTACCCCTGGCTCGTCGCCGAGGTGGAGGGCGT

CGTCGCCGGCATCGCCTACGCCGGCCCCTGGAAGGCCCGCAACGCCTACG

ACTGGACCGTCGAGTCGACGGTGTACGTCTCCCACCGGCACCAGCGGCTC

GGACTGGGCTCCACCCTCTACACCCACCTGCTGAAGTCCATGGAGGCCCA

GGGCTTCAAGAGCGTGGTCGCCGTCATCGGACTGCCCAACGACCCGAGCG

TGCGCCTGCACGAGGCGCTCGGATACACCGCGCGCGGGACGCTGCGGGCA

GCCGGCTACAAGCACGGGGGCTGGCACGACGTGGGGTTCTGGCAGCGCGA

CTTCGAGCTGCCGGCCCCGCCCCGCCCCGTCCGGCCCGTCACACAGATCT

GAGCGGAGAGCGCATGGC

FIGURE 9

```
                    10         20         30         40         50
PBG39------   MSPERRPADI RRATEADMPA VCTIVNHYIE TSTVNFRTEP QEPQEWTDCL
PJS1-------   VSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDCL

                    60         70         80         90        100
PBG39------   VRLRERYPWL VAEVDGEVAG IAYAGPWKAR NAYDWTAEST VYVSPRHQRT
PJS1-------   ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL

                   110        120        130        140        150
PBG39------   GLGSTLYTHL LKSLEAQGFK SVVAVIGLPN DPSVRMHEAL GYAPRGMLRA
PJS1-------   GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA

                   160        170        180
PBG39------   AGFKHGNWHD VGFWQLDFSL PVPPRPVLPV TEI*
PJS1-------   AGYKHGGWHD VGFWQRDFEL PAPPRPVRPV TQI*
```

FIGURE 10

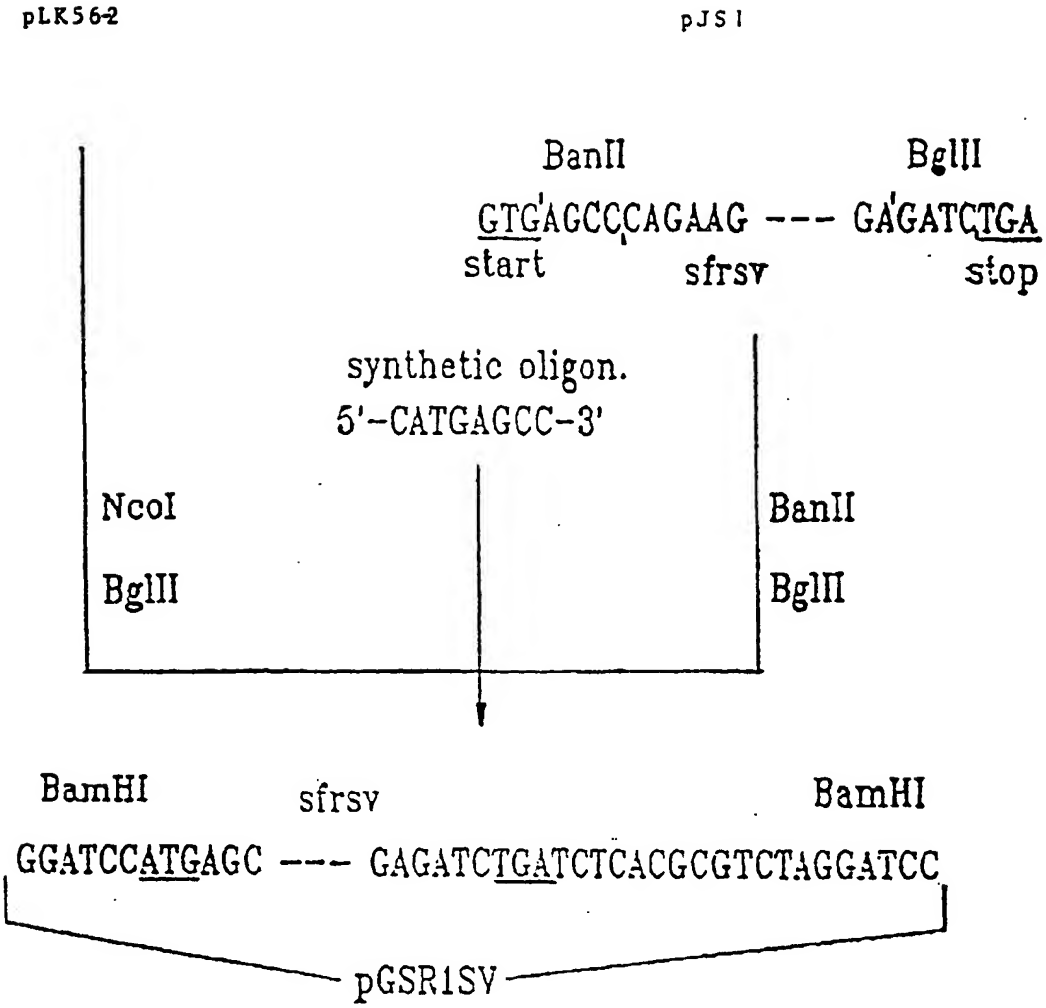

pLK56-2
pJS1
BanII
BglII
GTGAGCCCAGAAG --- GAGATCTGA
start
sfrsv
stop
synthetic oligon.
5'-CATGAGCC-3'
NcoI
BglII
BanII
BglII
BamHI
sfrsv
BamHI
GGATCCATGAGC --- GAGATCTGATCTCACGCGTCTAGGATCC
pGSR1SV

FIGURE 11